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UNIVERSITY OF ALBERTA

The use of Cytochrome *b* Sequence Variation to Assess Genetic Differences between Species and Subspecies of *Vireo* (Aves).

by

Brent W. Murray

A Thesis

Submitted to the Faculty of Graduate Studies and Research

in Partial Fulfilment of the Requirements for the

Degree of Masters of Science

Department of Zoology

Edmonton, Alberta

Fall, 1991

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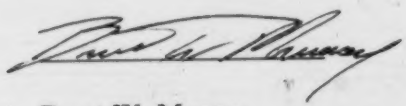
Signed 

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ABSTRACT

A recent phylogenetic study of the genus *Vireo* (Johnson *et al.* 1988), based on protein electrophoresis, has found the taxon to be polyphyletic. This estimate of phylogeny places confamilial species of the genera *Virolanius*, *Cyclarhis* and *Hylophilus* within the *Vireo* taxon questioning the traditional classification of the genera. In order to independently test these results, sequence data from the cytochrome *b* (cyt-b) gene have been collected from the following 9 *Vireo* species: Bell's (*Vireo bellii*), Gray (*V. vicinior*), Solitary (*V. solitarius*), Black-capped (*V. atricapillus*), White-eyed (*V. griseus*), Philadelphia (*V. philadelphicus*), Warbling (*V. gilvus*), Red-eyed (*V. olivaceus*) and Black-whiskered (*V. altiloquus*), and two confamilial species: Rufous-browed Peppershrike (*Cyclarhis gujanensis*) and Slaty-capped Shrike-vireo (*Vireolanius leucotis*).

For each of the above species at least 273 homologous base pairs from the cyt-*b* gene have been amplified using the polymerase chain reaction and sequenced. Phylogenetic estimates were achieved through both phenetic and cladistic analyses in which transversions and selected 1st and 2nd codon base pair substitutions were weighted higher than transitions. The evolutionary histories produced support the traditional monophyletic taxonomy and contradict Johnson *et al.* (1988).

In addition to the phylogenetic analysis two subspecies pairs (*V. gilvus gilvus, V. g. swainsonii* and *V. solitarius solitarius, V. s. cassinii*) were assessed for genetic differentiation. In both species, possible mtDNA introgression was observed between the subspecies. The average amounts of nucleotide substitutions, 8 and 13 respectively, were greater than the maximum number (2) of substitutions found within subspecies. This level of genetic differentiation is similar to that of the closely-related species *V. olivaceus, V altiloquus* (11) and *V. bellii, V. griseus* (15) and justifies further investigation into the rank of the taxa. These subspecies are distinct evolutionary units which have retained the ability to hybridize.

All substitutions leading to amino acid changes were assessed. Of the 12 amino acid changes observed, all were conservative in nature involving changes from nonpolar to nonpolar or to slightly polar groups and probably do not impart any functional significance. These observations support the conserved nature of this metabolically important protein.

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Chapter 1

INTRODUCTION

Vireo Systematics

The species classified within the genus *Vireo* are behaviourially and morphologically similar (Hamilton 1958, 1962). This similarity has led to uncertainties as to the relationships within the taxon. In 1988 an extensive electrophoretic survey was conducted by Johnson *et al.* in order to resolve these questions. Their study, however, raised new questions about the rank of taxa within the genus and even about the validity of the genus itself. Before drastic revisions are made to *Vireo* classification these results need to be examined carefully. In order to independently test the results of Johnson *et al.* sequence data from the mitochondrial gene cytochrome *b* will be used to reestimate the evolutionary history of the genus *Vireo*.

The New World passerine family Vireonidae is comprised of four genera; *Vireo, Hylophilus, Vireolanius and Cyclarhis*. The genus *Vireo* is the largest of these containing 26 species, (A.O.U. 1983) 11 of which are found in North America. A typical vireo is a small, greenish, song bird ranging in weight from 9 to 18 grams. Its is insectivorous, especially in the breeding season, and frugivorous (Barlow 1980). All vireos construct similar nests which are hung from two horizontal branches at the point of separation.

Traditionally the relationship of the genus *Vireo* to other avian families was unclear. Two main views were established. The similarity of their hooked bill to that of shrikes led the genus to be aligned with family Laniidae. Conversely, based on their size, coloration and the tendency toward the reduction of the 10th primary, the vireos were thought to be associated with the nine-primaried oscine assemblage, in particular the Wood Warblers (Parulinae). Recent molecular studies, however, have all supported a close relationship to a corvine assemblage, including Corvidae, Laniidae and others (Sibley and Ahlquist 1982; Johnson *et al.* 1988).

Based on a variety of morphological and behaviourial characters the genus *Vireo* is currently subdivided into three subgenera (*Vireo*, *Lanivireo* and *Vireosylva*) by the American Ornithologists' Union (1983). Vireo species (relevant to this study) are: Subgenus *Vireo* [Bell's Vireo (*Vireo bellii*), White-eyed Vireo (*V. griseus*) and Black-capped Vireo (*V. atricapillus*)], Subgenus *Lanivireo* [Gray Vireo (*V. vicinior*), Solitary Vireo (*V. solitarius*)], and Subgenus *Vireosylva* [Warbling Vireo (*V. gilvus*), Philadelphia Vireo (*V. philadelphicus*), Black-whiskered Vireo (*V. altiloquus*) and Red-eyed Vireo (*V. olivaceus*)]. At present the validity of the subgenus *Lanivireo* is in doubt with most authors placing its members within the subgenus *Vireo* (Barlow 1980; Johnson *et al.* 1988). This leaves two major clades within the genus. They are commonly known as the eye-ringed vireos (*Vireo* including *Lanivireo*) and the eye-lined vireos (*Vireosylva*).

The genus Vireo represents an interesting contrast in evolution. Although morphologically and behaviourially similar (Hamilton 1958, 1962) the species within the genus show a high amount of molecular divergence relative to other passerine congeners (Avise *et al.* 1982; Johnson *et al.* 1988). Johnson *et al.* (1988) reported a mean genetic distance (Nei 1978) of 0.29 within *Vireo*. This value is small when compared to nonavian vertebrates, but is three times larger than the mean genetic distance among the species of the genus *Anas* and at least four times larger than mean distances among species within the passerine genera, *Melospiza*, *Dendroica* and *Parus*, studied to date. (see Tegelstrom and Gelter 1990, for a review of genetic distances). This large distance may be due to two factors which are not mutually exclusive. The taxon may be relatively old (Avise *et al.* 1982) or the taxon has been undersplit at the generic and specific levels (Johnson *et al.* 1988).

Johnson *et al.* (1988) conducted an extensive electrophoretic survey of the family Vireonidae and reported the subgeneric divisions to be questionable. From evidence of protein variation, they conclude there are four main taxonomic groupings within the genus *Vireo* (see Figure 1). They find no evidence to support the subgenus *Lanivireo* as its member species are grouped consistently with those of the subgenus *Vireo*. Additionally, the subgenera *Vireo* and *Lanivireo* are shown to be more closely related to members of the confamilial genera *Vireolanius* and *Cyclarhis* than they are to the subgenus *Vireosylva* and to *V. bellii*. *V. bellii* appears to be the lone member of a unique taxon. This suggests a polyphyletic origin for the genus. Lastly, the subgenus

Vireosylva is broken into two groups; the *gilvus* group (*V. gilvus* and *V. philadelphicus*) and the *olivaceus* group (*V. olivaceus*). Consistent with the hypothesis that the genus *Vireo* is polyphyletic and therefore undersplit, Johnson *et al.* suggest that the four taxonomic groupings be raised to generic rank. They declined, however, to make this a formal recommendation. They do recommend formally the disuse of the categories of subgenera within *Vireo*.

Molecular Analysis

The use of molecular characters for estimating phylogenies has expanded greatly in recent years. Molecular variation can be quantified in three main ways; protein electrophoresis, restriction fragment analysis or direct DNA sequencing. Protein electrophoresis involves the examination of genetic variation by analysis of the product of DNA expression. Proteins are characterized by their migrational traits in the presence of an electrical field. Utilizing this technique a great number of loci can be examined. The major drawbacks to this type of study are that: (a) a large amount of genetic variation not directly involved with protein coding is unobtainable; and (b) the homology of the characters, electromorphs, is uncertain as similar charge changes at different amino acid positions may lead to identical electromorph mobilities. This makes cladistic analyses of the data unreliable. Direct characterization of the DNA is required to insure character homology.

Restriction site analysis is a common method of DNA characterization. In this technique a given region of DNA is studied in every sample. This region is digested with various restriction enzymes which recognize specific palindromic sequences and then cleave the DNA. By electrophoretic analysis of the resulting DNA fragments, restriction maps can be created and compared. Phylogenetic inferences are drawn by analyzing site losses and gains. Although this method represents an improvement in the resolution of homology over protein electrophoresis, a large amount of DNA variation still remains undetectable. Only by direct DNA sequencing will all the variation be available for phylogenetic analysis. As opposed to restriction fragment analysis and protein electrophoretic studies no variation will remain hidden and direct estimates of DNA divergence can be achieved.

Until recently the first two methods were the most widely used. DNA sequencing traditionally involved time-consuming methods utilizing repeated steps of cloning and sequencing. With the advancement of polymerase chain reaction (PCR) (White *et al.* 1989) technology, it has become feasible to carry out population surveys of molecular variation by the sequencing of specific genes. PCR technology involves the amplification of selected regions of DNA through the use of region specific oligionucleotide primers, thermal cycling and the heat stable Taq polymerase. This amplified product can be isolated easily and then sequenced directly.

The appropriate region of DNA to consider in a phylogenetic study is a function of the relatedness of the taxa examined. For phylogenetic inferences between kingdoms, highly conserved regions such as ribosomal DNA are desirable. Conversely, mitochondrial DNA (mtDNA) is ideal for phylogenetic analysis of closely-related taxa because it evolves about 10 times faster on average than nuclear DNA (Brown 1985). In addition, it is a haploid, maternally-inherited, genome which makes it easier to characterize than diploid, nuclear DNA. For example, only one product will be amplified during the PCR reaction. Haploidy simplifies the characterization (*i.e.*, sequencing) of the molecule as there is only one allele per individual.

In the class Aves, mitochondrial DNA variation has been used to examine species relationships within genera and within species, e.g., waterfowl (*Anas*), sparrows (*Melospiza*), warblers (*Dendroica*) (Kessler and Avise 1985), geese (*Anser*) (Shields and Wilson 1987), chickadees and titmice (*Parus*) (Mack *et al.* 1986), sparrows (*Ammodramus*) (Zink and Avise 1990), flycatchers (*Ficedula*) (Tegelstrom *et al.* 1990), Australian rosellas (*Platycercus*) (Ovenden *et al.* 1987) and Australian babblers (*Pomatostomus*) (Edwards and Wilson 1990). Various sibling species have also been examined for mtDNA variation (Avise and Zink 1988). At the subspecific level mtDNA variation has also been characterized for the Canada Goose (*Branta canadensis ssp*) (Shields and Wilson 1987), the Tufted Titmouse (*Parus bicolor ssp*) (Avise and Zink 1988), Australian rosellas (*Platycercus elegans ssp* and *P. adscitus ssp*) (Ovenden *et al.* 1988), an Australian babbler (*Pomatostomus temporalis ssp*)

(Edwards and Wilson 1990) and the Northern Flicker (*Colaptes auratus ssp*) (Moore *et al.* 1991). The Northern Flicker study represents the single most detailed geographic survey of mtDNA variation conducted in an avian species to date. Other large geographic studies of mtDNA variation include surveys of the Red-winged Blackbirds (*Agelaius phoeniceus*) (Bali *et al.* 1988), the Fox Sparrow (*Passerella iliaca*) and the Song Sparrow (*Melospiza melodia*) (Zink 1991).

In all of the above studies, except Edward and Wilson, 1990, mitochondrial variation was characterized through restriction fragment analysis. Edwards and Wilson, 1990, used cytochrome *b* sequence variation to estimate genetic variation. Cytochrome *b* is essential for the proper transfer of electrons in the electron transfer chain. It is one of the 9-10 proteins that combine to form complex III of the mitochondrial oxidative phosphorylation system (Hatefi 1985). This complex transfers electrons from dihydroubiquinone to cytochrome *c* which is coupled with the translocation of proteins across the inner mitochondrial membrane (Hatefi 1985). Cytochrome *b*, which is a membrane bound protein, is one of the most conserved regions of the mitochondrial DNA (Desjardins and Morais 1990).

The complete sequence and mitochondrial position of cyt-*b* in birds (see Figure 2) has been characterized only in chickens (Desjardins and Morais 1990). It is a protein of 380 amino acids in length, located not adjacent to the D-loop, as in mammals, but is separated from it by the ND6 gene.

Study Objectives

As an independent test of the results of Johnson *et al.* (1988) sequence data from the mitochondrial cytochrome *b* gene are used to reconstruct an estimate of the phylogenetic history of the genus. For this study only 91 amino acids are compared. The region containing these 91 amino acids is known to be a variable component of the gene (Kocher *et al.* 1989; Irwin *et al.* 1991). The results of the two independently derived phylogenies are compared directly. The following taxa within the genus *Vireo* were examined; *Vireo gilvus gilvus, V. g. swainsonii, V. solitarius solitarius, V. s. cassinii, V. philadelphicus, V. olivaceus, V. altiloquus, V. atricapillus, V. vicinior, V. bellii, and V. griseus.* These species represent each of the four major clades described by Johnson *et al.* (1988). In addition the two confamilial species *Vireolanius leucotis* and *Cyclarhis gujanensis* are analyzed. These species allow the postulated polyphyletic origin to be examined. Polyphyly would be demonstrated if either of these species should group with the eye-lined or the eye-ringed clades.

The species within the genus *Vireo* exhibit a high amount of molecular divergence and yet are similar both morphologically and behaviourially. It may, therefore, be reasonable to assume that subspecies within the genus, which are easily characterized, may also show relatively high degrees of molecular differentiation. By examining the variation which exists between such subspecies, insight into their taxonomic rank may be gained. Within the province of Alberta two subspecies pairs are easily identifiable

on the basis of song type and plumage differences. They are for the Warbling Vireo, the eastern form *Vireo gilvus gilvus* and the western form *V. g. swainsonii*, and for the Solitary Vireo, the eastern form *Vireo solitarius solitarius* and the cordilleran form *V. s. cassinii*. Both these subspecies pairs display the typical eastern *vs* western distributions characteristic of a large number of closely-related avian taxa (Rising 1983) (see Figures 3 and 4). In addition to the characterization of cytochrome *b* variation presented here, these populations are currently being examined for morphological, song type and allozyme variation. The amount of mitochondrial variation will be a vital component in the overall study of population differences.

In addition to their use in a phylogenetic and subspecific analyses, all amino acid changes discovered will be examined as to their possible effect on the cyt-*b* molecule. The variable amino acid positions will be assessed as to their nature of change and location in the molecule.

Chapter 2

MATERIALS AND METHODS

Sample Collection

Tissue samples representing 9 *Vireo* species commonly found in North America and for two species of the confamilial genera *Vireolanius* and *Cyclarhis* were collected during the 1988 to 1990 field seasons. For each sample, liver, heart, and pectoral muscle were either frozen on dry ice or in liquid nitrogen following collection and stored in a -70 ^{0}C freezer. All samples were acquired by either the Provincial Museum of Alberta or the Royal Ontario Museum. The species collected, sample sizes, geographic location of the acquisitions, and corresponding haplotypes are listed in Table 1. For the subspecific analyses 4 to 6 samples per subspecies were collected in either Alberta or Saskatchewan. The approximate species distributions in this area and the sample locations are shown on Figures 3 & 4.

Extraction of Total DNA

DNA was extracted as either total DNA or mtDNA. Total DNA was extracted as follows. Two grams to as little as 50 mg of frozen tissue was placed in 5 to 0.5 ml of ice cold L-medium (see Appendix A for all unspecified solutions) and allowed to thaw on ice. The sample was then homogenized twice using a Brinkmann Homogenizer,

model pt 10/35. The first homogenation was done gently for 15 seconds, i.e., conducted at low speeds, 3 on a scale from 1 to 10. After this the sample was left on ice for 10 minutes and then homogenized at a faster speed, i.e. 4, for 15 seconds. The point of this step is to break open the cell while leaving the organelle membranes intact. After homogenation the sample was lysed by the addition of a concentrated Protease K solutions to achieve a working concentration of 50 µg/ml and a 20% SDS solution to achieve a final concentration of 1% SDS. The lysis solution was left at 4 ^{0}C overnight and then incubated for 2 hours at 37 ^{0}C. At this point a number of phenol extractions were conducted to remove proteins, lipids and any other contaminants. In each case an equal volume of phenol was added to the lysed sample. This solution was then mixed by gentle inversion and centrifuged for 10 minutes at 6000 rpm in a Beckman model J2-21M/E centrifuge using a JA20 rotor. After the centrifuge the lighter solution containing the DNA was drawn off and the heavier phenol layer discarded. This step was repeated to the DNA solution for up to four times or until the sample became clear. At this point an additional phenol\chloroform (see appendix A) and a chloroform extraction were conducted in a manner identical to that described above. The DNA sample was then precipitated by adding 2 volumes of 95% ethanol and a half volume of 7M ammonium acetate. This solution was mixed well and then centrifuged for 30 minutes at 10,000 rpm AT 4 ^{0}C. The remaining pellet was wash in 70% ethanol, dried in a desiccator and resuspended in TE buffer.

Extraction of mtDNA

An alternate approach to total DNA extraction was to extract only the mitochondrial DNA. The alkaline lysis method described by Tamura and Aotsuka (1988) provides a quick and effective method of extracting mtDNA which is suitable as a substrate for the polymerase chain reaction. The following protocol is a variation of the above alkaline lysis technique. One gram of sample was homogenized in 10 ml of H-medium in the same manner as the total DNA extraction. This solution was then centrifuged at 3500 rpm for 3 minutes at 4 ^{0}C in order to spin down the intact nuclei and other cellular debris. At this speed the mitochondria remain in solution. This supernatant is recovered and subsequently centrifuged at 10,000 rpm for 10 minutes at 4 ^{0}C to pellet the mitochondria. The remaining solution is removed and the mitochondrial pellet is resuspended in 1 ml of ice cold solution I. To this solution 2 ml of freshly prepared room temperature solution II is gently mixed. This solution is placed on ice for 5 to 8 minutes. After this time 1.5 ml of ice cold solution III is added, the solution is gently vortexed and placed on ice for an additional 5 minutes. Next the solution is centrifuged at 10,000 rpm for 5 minutes at 4 ^{0}C. The supernatant containing the mitochondrial DNA is transferred to a clean tube and phenol/chloroform extracted twice, as described previously. MtDNA is precipitated by the addition of 2 volumes of 95% ethanol only. No salt is required as solution III provides the high molarity for efficient DNA precipitation.

Amplification of Cytochrome b

Both total DNA or mtDNA work equally well as substrates for the enzymatic amplification of cytochrome *b*. One to 2 µl of sample representing about 100 ng of DNA was used to start the reaction. The reaction mixture contained 10 µl of a 10x standard PCR buffer (2.0 mM $MgCl_2$ final concentration), 200 mM dNTP's, 1 unit of Taq DNA polymerase (Promega), 20 pmol of each primer, was made up to a volume of 100 µl with deionized H_2O and was covered by a layer of oil to prevent condensation. The primers are H15149 (5' > CCTCA GAATG ATATT TGTCC TCA < 3') and a modified version of L14841 (5' > ATCCA ACATC TCAGC ATGAT GAAA < 3') as described by Kocher *et al.* (1989). The modification involved the removal of 5 to 6 base pairs at the 5' end. A water cooled Techne PHC-2 thermal cycler was used to vary the temperature in the reaction. The program for the thermal cycler was as follows. The first cycle heated the sample up to 93 ^{0}C for five minutes to denature the DNA and then cooled the sample to 54 ^{0}C holding it there for one minute to anneal the primers to the cytochrome *b* templates. The last step in the cycle is to heat the sample to 72 ^{0}C. At this temperature the taq poylmerase is most active and replication of DNA occurs. This initial cycle is followed by a slightly different cycle which is repeated 30 times. During these cycles the template DNA is amplified considerably. This cycle heats to 94 ^{0}C for 15 seconds, cools down to 54 ^{0}C for 30 seconds, and then raises the temperature to 72 ^{0}C for 2 minutes. The final cycle is identical to the previous except the sample is held at 72 ^{0}C for 10 minutes to ensure

that all the polymerization reactions have completed insuring the final product is double stranded. For reviews on PCR optimization see Innis and Gelfand (1990) and Saiki (1989).

Isolation of PCR product

In order to insure that the desired PCR product is sequenced it is isolated from any of the possible secondary products. This is accomplished by electrophoresing the sample on a 1% low melting point agarose gel to separate the products according to size. The desired product is cut out of the gel under long wave UV light so as not to nick the DNA. The LMP wedge is dissolved in deionized H_2O up to a volume of 1 ml by heating for 10 minutes at 65 ^{0}C (Gyllensten 1989). This solution is then used as the template for asymmetric amplification of the product. For each sample two separately produced products were obtained. In every case for the cytochrome *b* amplifications only one product was visualized on the gels.

Asymmetric Amplification

Asymmetric amplification results in the production of two products one double stranded and one single stranded. The single stranded product is the object of this reaction as it is required for sequencing. Sequencing of double stranded products under 500 base pairs is complicated due to the rapid reannealing of the complimentary

strands. This reannealing is so rapid that it effectively competes with the annealing of the primers needed for sequencing (Gyllensten 1989). The production of single stranded DNA is facilitated by the limiting of one of the primers to a low concentration. Thus, when this primer is used up, the reaction will switch to the production of single stranded DNA. The reaction concentrations were as follows: 40 pmol of one primer, 0.5 pmol of the other primer, 100 mmol dNTP's, 10 µl of a 10X asymmetric reaction buffer (0.5 mM $MgCl_2$ final concentration), 2 units of Taq polymerase, and H_2O up to a final volume of 100 µl. The solution was covered by 75 µl of mineral oil and contained in a 500 µl microcentrifuge tube. The cycles for the asymmetric reaction were similar to the initial PCR except the middle amplification cycles were repeated 40 times. The initial and final cycles were identical. For reviews on the optimization of asymmetric PCR see McCabe (1990) and Gyllensten (1989).

For every sample, one of the two isolated products was used as a template to produce a specific single stranded DNA while the other product was used as a template to produce the other complimentary strand. In this way any errors of the PCR reaction can be identified during sequencing. After the PCR reaction DNA was concentrated for sequencing by combining two reactions, each of which was started from the same initial PCR template, in a 1.5 ml mircocentrifuge tube. To this solution an equal volume of phenol/chloroform was added, mixed gently and then centrifuged for 3 minutes in an epindorf microcentrifuge (model 5414, one speed approximately 13,000

rpm) to separate the phases. This step cleans out any impurities and residual oil. The DNA was precipitated in 2 volumes of 95% ethanol and a half volume of 7M ammonium acetate. This solution is left at -70 ^{0}C for at least 3 hours and then centrifuged for 30 minutes in the epindorf microcentrifuge. The pellet was dried and reprecipitated in 13 µl of deionized H_2O.

To quantify the product 3 µl of this product was added to 7 µl of 1X loading buffer and electrophoresed on a 1% agarose gel in a half times TBE buffer. The gel was subsequently stained with 10 µl of ethidium bromide (EtBr: 10 mg/ml) in 50 ml of H_2O for one hour. The gel was stained subsequent to the electrophoresis because the EtBr affects the migration of the single stranded DNA through the gel in relation to the double stranded DNA of similar length. With no EtBr present the single stranded product migrates more slowly than double stranded. Alternatively with EtBr present the single stranded DNA migrates at a faster rate than double stranded DNA. This fact can lead to confusion in interpreting results.

Sequencing of the Asymmetric Product

Once the presence of single stranded product was assured, the sample was ready for sequencing. Two different kits were used for this reaction. The US Biochemical Sequenase kit Version 2.0 and the Pharmacia ^{T7}sequencing kit. Both of these kits employ the Sanger dideoxey chain termination method of sequencing. In each case

the primers were annealed by addition of 0.5 pmol of the appropriate primer and 2 µl of the annealing buffer. This solution was heated to 65 ^{0}C for 10 minutes and allowed to cool at room temperature for 30 minutes. The only deviation made from the recommended protocols was that no elongation step was carried out. The mixture of primed template, S^{35} labelled dATP (Nen, research products, Dupont), reaction buffer and sequenase Version 2.0 (T-7 polymerase) were added directly to the termination mixes at which point the reactions commenced. This reaction was allowed to proceed for 5 minutes after which it was halted through the addition of stop buffer. For a complete list of the solutions used, see the manuals contained within the appropriate kits.

Sequencing products were separated on 6% denaturing acrylamide gels. This gel was electrophoresed in a BRL vertical gel apparatus. 1X TBE buffer was placed covering the top of the gel while 1X TBE plus 1 M sodium acetate was covering the bottom of the gel. The high salt concentration slows down the migration of the smaller bands and thus increases the range of readable bands (Ausubel *et al.* 1989). The gel was preheated by running current through them maintained at 70 watts for 30 minutes. After this the gels were loaded with labelled DNA denatured by heating at 100 C for 2 minutes and placed directly on ice. The gel was then electrophoresed for 2 or 3 hours, depending on the area of sequence to be analyzed, at 70 watts constant power. Once completed, the gel was washed and fixed by immersion in 5% methanol and 5% acetic acid for 10 minutes. The gel was now transferred to Whatman paper and dried. The

dried gel was then exposed to X-ray film and developed in three days (longer exposure was carried out when necessary).

Phylogenetic Analysis

The nucleotide substitutions were examined for their phylogenetic content in two separate manners. All phenetic analyses were performed with the aid of the computer package Phylip 3.3 (Felsenstein 1990). The program DNAdist was used to calculate corrected DNA distance values based on Kimura's "2-parameter" model (1980). A default setting of a 2 to 1 rate of transition to transversion substitutions was used to create the values seen in the upper diagonal of Table 3. These values represent a corrected distance measure ranging from 0 to 1. The distance matrix was then used to create the distance trees (Figures 5 and 6). The tree in Figure 5 was constructed using the Kitsch program included in the Phylip package. It utilized the Fitch-Margoliash tree building methods under the assumption that an evolutionary clock is in effect. Figure 6 was produced through a similar method using the Fitch program. This program utilizes the same tree building methods but does not assume the presence of a molecular clock. Bootstrap analyses were carried out using the bootstrap options available in the programs. In each analysis 100 bootstrap data bases were created from which trees were constructed. A consensus tree of the bootstrap trees was made using the program Consensus which constructed a majority rule tree. This program produces a consensus tree that consists of all groups that occur most often in the

replicates. Although it is a majority rule tree, branch points found less than 50% of the time will be shown if contradictory branches of equal value do not exist.

Cladistic analyses were performed with the computer programs PAUP 3.0 (g) (Swofford 1989) and MacClade 2.1 (Maddison & Maddison 1987). The most parsimonious trees found by PAUP using unordered fully reversible characters (Wagner parsimony) were further analyzed by MacClade to gain a better understanding of the character evolution. In both programs transversional and selected 1st and 2nd codon base pair changes were given extra weight through the use of weighted characters and stepmatrices.

Chapter 3

RESULTS

Cytochrome b Sequence Evolution

At least 273 homologous base pairs of a region of the mitochondrial cytochrome *b* (Cyt-*b*) gene were sequenced for each of the 32 samples listed on Table 1. Within these 32 samples 14 species or subspecies were examined and 20 haplotypes found (see Table 2). Ninety-six base pair changes were found in 78 sites in the 19 haplotypes identified for the Family Vireonidae. Eleven, 4 and 81 of the these changes occurred in first, second and third base pairs of codons respectively. Of the 96 total base pair substitutions, 72 were transitions, 41 of which are shared between taxa and thus phylogenetically informative; 24 were transversions, 18 of which are informative. No other mutational events such as insertions or deletions were observed. In every sample the cyt-*b* gene coded for a complete protein segment of the gene with no stop codon encountered.

Intraspecific Variation of Haplotypes

Information about intraspecific variation is limited to the four vireo species with sample sizes greater than one: *V. bellii* (2), *V. olivaceus* (3), *V. solitarius ssp* (9) and *V. gilvus ssp* (11). Both the Bell's and Red-eyed Vireos display similar levels of intraspecific variation with at most 2 nucleotide differences. The maximum number of nucleotide

differences between individuals is larger for the Solitary and Warbling Vireos: 13 and 9 respectively. If, however, variation within subspecies of the species is examined, with all possible instances of haplotype introgression ignored (see next 2 paragraphs), the number of nucleotide differences is similar to those seen in the Bell's and Red-eyed Vireos. No variation was observed between the haplotypes of the western subspecies *V. s. cassinii* and *V g. swainsonii* while 2 nucleotide substitution are observed for both the eastern subspecies *V. s. solitarius* and *V. g. gilvus*.

Evidence of possible cyt-*b* haplotype introgression was observed in both subspecies pairs examined. In both surveys a haplotype form, either identical or very similar to the form most often associated with subspecies 'A', was found in a sample classified on morphological grounds as subspecies 'B'. Two such instances occurred in the *Vireo gilvus ssp.* In the first instance a *Vireo g. gilvus* sample collected near Beaverhills Lake in Alberta (Figure 3) has the exact haplotype found in 5 out of 6 of the *Vireo gilvus swainsonii* samples. The only *Vireo g. swainsonii* sample that does not contain this haplotype was collected in Sundre, Alberta. Unfortunately this sample suffered some degradation before processing and did not prove ideal for PCR amplification. I have chosen to mention this sample because it appears to have the a haplotype very similar to those most often associated with *Vireo g. gilvus* samples. Because of the incomplete data on this sample it has been excluded from all phylogenetic analyses. Possible haplotype introgression is also observed in the *Vireo solitarius ssp.* A *Vireo s. solitarius* sample collected along the Clearwater River (Figure 4) was found to possess the only haplotype

found in all *Vireo s. cassinii* sampled. This sample is of interest because although it was classified *solitarius* on the basis of plumage, it was noted in the field to have a song type more like *cassinii* populations (J.C. Barlow and W.B. McGillivray, unpublished data).

Phenetic Analysis

For both the cladistic and phenetic analyses, the introgressed haplotypes were ignored. The most common haplotypes for each subspecies were used as representatives of the populations. Using this approach, it is clear that the differences between the main haplotypes of one subspecies are much smaller than those between the main haplotypes of the subspecies pairs (see Figure 5 and 6). The difference between the haplotypes of *Vireo g. gilvus* is the result of at the most 2 base pair changes or 0.0074 (Kimura's distance, Table 3). In contrast an average of 8 mutational changes (0.0298) are found between the *Vireo g. swainsonii* haplotype and the *Vireo g. gilvus* haplotypes. A similar situation is also observed in the *Vireo solitarius ssp.* In this comparison the distance between the subspecific haplotypes is even larger averaging to 0.0489 (13 mutational changes). This value is very similar to distance values found between other closely related species such as *Vireo olivaceus* and *Vireo altiloquus* (0.0450) and *Vireo bellii* and *Vireo griseus* (0.0568). The pair-wise species distance within the genus *Vireo* averages 0.0941 and ranges from 0.0450 to 0.1284.

Two distinct distance trees were constructed using the computer programs Kitsch and Fitch (Figures 5 and 6 respectively) contained within the phylogenetic package Phylip 3.3 (Felsenstein 1990). The main difference in the programs is that Kitsch assumes a molecular clock is in effect when constructing a tree. All the branches are assumed to be evolving at a similar rate and thus the tips are contemporaneous. In both trees, the confidence values of the internal lineages was assessed by a bootstrap analysis (Felsenstein 1985). The values at each node indicate the number of trees out of 100 that possessed this branch. Ninety five trees out of 100 is considered as a significant level of confidence. What the bootstrap really measures is the consistency of characters. The more consistent the characters, the higher the confidence value.

There appear to be two major clusters in the Kitsch distance tree (Figure 5). The first contains all eye-lined Vireos sampled (subgenus *Vireosylva*) while the second contains all the eye-ringed Vireos (subgenera *Vireo* and *Lanivireo*). Next, every *Vireo* sample clusters together with the confamilial species of *Cyclarhis gujanensis* and *Vireolanius leucotis* being outliers. As mentioned previously the bootstrap values are accepted as confidence levels. In this tree bootstrap values are considerably lower for the deeper branch points. Only the relationships between individuals of the same species and between *V. gilvus* ssp can be viewed with confidence. An '*' indicates the node was not present in the majority rule bootstrap tree.

The Fitch distance tree (Figure 6) is similar to the Kitsch tree (Figure 5) in many respects. There is still a clear distinction between the eye-lined and eye-ringed vireos. The two major differences involve the placement of *V. philadephicus* and *Cyclarhis gujanensis*. *V. philadelphicus* is placed closer to the *V. gilvus ssp* and *Cyclarhis gujanensis* is internal to the Vireo clade aligning closely with the Solitary Vireos. In both trees the confidence of these branches is very low.

Cladistic Analysis

Figure 7 represents a majority rule consensus tree of 43 parsimony trees requiring 186 mutational changes or less. These trees were constructed using all informative characters in an unweighted data set and were found using the heuristic searching method of the computer program PAUP version 3.0 (g) (Swofford 1989). The numbers along the branches indicate the percentage of trees that agree with the branching pattern. The numbers in brackets at the nodes indicate the confidence values found by a bootstrap analysis. Only values greater than 50% are shown. This tree is very similar to Figure 6 and in fact has the same drawbacks. The confidence in the position of the deeper branch points is very low.

As mentioned previously, the significance of a bootstrap analysis is directly related to the consistency of the characters analyzed. In Figure 7 every character is taken to be equally informative. This situation is probably not true. In this data set there are three types of

characters; silent transitions, silent transversions and 1st and 2nd codon changes that lead to amino acid changes in the protein. Other investigators have estimated that the ratio of silent transitions to silent transversions ranges from 10:1 to 20:1 (Aquadro and Greenberg 1983; Brown 1985; Edwards and Wilson 1990). This range is supported by the data presented here. Between closely-related species, e.g., *V. olivaceus / V. altiloquus* (11 transitional changes), *V. bellii / V. griseus* (14 transitions and 1 transversion) and *V. gilvus ssp / V. philadelphicus* (20 transitions and 1 transversion), a single transversion is accompanied by at least 14 transitions (Table 3). It appears that transversions are accumulating at a much slower rate and therefore should be more informative phylogenetically than transitions. Studies of the mitochondrial rRNA of artiodactyls and mammalian cytochrome *b* have shown that transversions are accumulating in a linear manner over time scales much greater than those considered here (Miyamoto and Boyle 1989;Irwin *et al.* 1991). The rapid rate of transition accumulation quickly clouds their linear trail with parallel and reversion events once a plateau of divergence is reached (Irwin *et al.* 1991). As a result of these differing rates of accumulation many investigators have used transversions, and 1st and 2nd codon changes, exclusively in the construction of their trees (Meyer and Wilson 1990; Irwin *et al.* 1991).

To explore the merits of a weighting scheme favouring transversions over transitions, a subset of 38 sites that contain only informative transitional events was examined using the branch-bound search method of PAUP. Thirteen equally parsimonious trees were revealed. The consistency index (C.I.) for these trees was 0.421 (ranges from 0 to 1; 1

equals all characters 100% consistent for the tree shown). This index is low and indicates that a large number of reversal or parallel transitional events have occurred. The random weighting of these as occurs in a bootstrap analysis would result in a large number of incompatible trees and is probably the reason for the low level of confidence at deeper branch points revealed thus far. In a second analysis the subset of the 20 informative transversions and of selected 1st and 2nd codon changes were utilized. Twenty-six equally parsimonious trees were found requiring 37 mutational steps or less (1 tree was found to require 36 steps). These trees all have a C.I. of approximately 0.6. The majority rule consensus tree in Figure 8 summarizes these results. The bold numbers on the branches indicate the percent of parsimonious trees that contain this branching pattern. The bracketed number at the node is the percent of trees in a bootstrap analysis (from 400 trees) that show the same branching pattern. Although only the branching pattern within the eye-lined Vireo clade is supported by the bootstrap analysis the consistency of all the characters together is higher than in the transition analysis. From this result it can be argued that the transversional events contain a more consistent record of the phylogeny of the species under examination.

Additional cladistic analyses were performed utilizing the entire informative data set in which transversional events were given the extra weights of 2, 4, or 8. The entire data set was utilized because the transitions still contain some information about the more recent branch points. In this way it was hoped the ambiguities found within the eye-ringed group would be removed.

Figure 9 represents the single most parsimonious tree found when the transversions and 5 of the 8 (justified in amino acid analysis, 1st paragraph), phylogenetically informative, 1st and 2nd codon base pair changes received double weight. In this tree a monophyletic *Vireo* clade is evolving from a stem and the eye-lined vireos are the most recently derived. When the weights are doubled to 4 and again to 8, a different parsimonious tree is evident (Figure 10). As the tranversional events receive more importance, the topology of the tree changes. This is evident as the C.I. of the transitional changes is sightly lower in Figure 10 as it is in Figure 9. Figure 10 displays a slightly different history than Figure 9. Here a monophyletic *Vireo* clade is radiating in two distinct lineages. Again there is a clear distinction between the eye-lined and eye-ringed Vireos. The branching arrangement of the eye-lined Vireos is identical in Figures 7, 8, 9 and 10. A major point of ambiguity is the placement of *V. vicinior* and *V. atricapillus* within the eye-ringed clade. The *V. solitarius ssp* and the species *V. griseus* and *V. bellii* group consistently in pairs. Figure 10 is thought to represent the best approximation of the phylogeny of this clade because of the combined use of weighted and transitional characters. A bootstrap analysis was not performed on the weighted trees due to the exceedingly high amount of computer time needed. However, the bootstrap analyses in Figures 7 and 8 can be extrapolated to these trees since the same data sets (or portions of) were used to construct all trees.

Amino Acid Analysis

In these taxa, a total of, 16 1st and 2nd codons display base pair changes which lead to 12 amino acid changes (Figure 11). Six of these amino acid changes are phylogenetically informative. The conservative change of valine to isoleucine occurs in 3 of these informative amino acids changes (see Figure 11). Both valine and isoleucine contain nonpolar aliphatic side chains and are relatively unreactive. Furthermore in the molecule studied, they all occur in transmembrane regions (Howell 1989), are all the result of transitions and, in the case of position 7, are highly variable. At this position, the change is found within one subspecies (*V. g. gilvus*) and also between both of the subspecies pairs examined. For these reasons, this amino acid change did not receive additional weights in the cladistic analyses. The remaining 3 informative positions, caused by 5 nucleotide substitutions, were either transversions or transitions that lead to a change from a polar to nonpolar amino acid. These changes were weighted as described above in the cladistic analyses.

In total, 12 amino acid positions were variable between the taxa examined (Figure 11). Of these, 7 are in transmembrane regions and primarily involve conservative changes between nonpolar amino acids. The one exception involves a change from threonine to alanine. Threonine has an aliphatic side chain which contains a hydroxyl group, is relatively unreactive and can exist in polar and nonpolar states (Creighton 1983). This amino acid change probably would not have a major effect on the molecule. Of the 5

extramembrane region substitutions the majority (4) occur in the region next to transmembrane region I. These changes are relatively conservative involving changes between the slightly polar threonine (discussed above), the closely related serine and various nonpolar groups. The most conserved area of the molecule appears to be transmembrane region II and the region involved in the Q^0 reaction centre (1 of 4 regions postulated in Howell, 1989). Only the single conservative amino acid change, leucine to isoleucine is observed in these regions. This change probably has no functional significance for a similar change is also observed in mammals (leucine to methionine, Irwin *et al.* 1991). In summary, all the amino acid changes are relatively conservative in nature and support the conserved nature of the protein.

Chapter 4

DISCUSSION

Subspecific Variation

For both subspecies pairs examined, there are Kimura distance differences between the main haplotypes of each the subspecies. The averages of 0.0298 for the *V. gilvus ssp* and 0.0450 for the *V. solitarius ssp* are similar to the 0.032 distance found between the subspecies *Pomatostomus temporalis temporalis* and *P. t. rubeculis* of the Australian Babbler (Edwards and Wilson 1990). They concluded that this high amount of variation between babbler subspecies warranted additional investigations into the specific status of these taxa.

Relatively few avian populations have been sequenced for cyt-*b*, therefore, comparisons of the *Vireo* data to that of other species are limited. However, differences between babbler subspecies were compared not only by sequencing cyt-*b*, but also through analysis of restriction site variation for the entire mitochondrial DNA molecule. Based on the restriction fragment analysis Edwards and Wilson reported an estimated distance of 8.2% between their subspecies for the entire mtDNA molecule. Compared with most North American avian taxa an intraspecific distance of about 8% is high and would place these babbler subspecies at the species rank (see Tegelstrom and Gelter 1990 for a review of genetic distances). On the assumption that the large differences between *Vireo* subspecies in cyt-*b* sequences is representative of the entire

mtDNA molecule, then an estimate of a minimum of 8% for total mtDNA divergence would be a reasonable approximation. This assumption, albeit tenuous, allows a comparison of the vireo intraspecific variation to the larger body of avian mtDNA restriction site variation data. The implication of this comparison, as with the babblers, is the possible raising of the subspecies to species rank and justifies a further investigation into the specific status of the Warbling and Solitary Vireo subspecies.

The evidence of introgressed haplotypes is most likely the result of hybridization events and not the presence of variation within a single large population. The strongest evidence for this view is in the amount of variation found between the haplotypes that has been discussed previously. The maintenance of two distinct and highly divergent forms in a single randomly mating species is highly unlikely and unprecedented in any other avian species examined. Studies of Red-winged Blackbirds and Northern Flickers have revealed little mtDNA variation over wide geographic ranges (Ball *et al.* 1988; Moore *et al.* 1991).

Further evidence that hybridization is occurring between distinct populations is seen in the birds themselves. The *V. s. solitarius* specimen that contains the *V. s. cassinii* haplotype has a mixture of vocal and morphological characters and was collected in an area where hybrids should be found if these populations are interbreeding (see Figure 4). The situation is less clear in the *V. gilvus ssp.* The sample collected at Sundre is clearly close to the population boundaries, however the bird collected in Beaverhills Lake is not (see Figure 3). This introgression may have occurred in an earlier

generation. This is supported by the morphological evidence which undoubtedly places the bird in *V. g. gilvus*. (W.B. McGillivray, unpublished data). Another alternative is that the putative donor may have come from a *V. g. swainsonii* population nesting in the closely situated Elk Island National Park. This area is prime *V. g. swainsonii* habitat and was once part of the ancestral range of the population. Due to a prohibition on collecting in Elk Island National Park the Warbling Vireo populations there have not been characterized.

Based on the cyt-*b* variation, in both the Warbling Vireo and Solitary Vireo samples examined, two distinct populations are evident between which hybridization still occurs. In a biological species concept, the evidence for hybridization supports the present subspecific status. However, much further work would be needed to support this view. The degree of hybridization should be monitored. In addition the viability, fertility and mobility of the hybrids should be studied to assess the amount of gene flow. In geese, mitochondrial DNA variation has been found between subspecies (Shields and Wilson 1987) in contrast to low nuclear DNA variation (Van Wagner and Baker 1986). A theory of high male dispersal and a female tendency to return to ancestral mating grounds has been used to explain this contrast. Although this situation seems unlikely for *Vireo* it can not be ruled out at present. Protein electrophoretic studies currently underway by Jon Barlow will be helpful in assessing nuclear DNA variation.

Alternatively, in a phylogenetic species concept (see McKitrick and Zink 1988), for which molecular data are more suited, the ability to hybridize is considered as an ancestral character which has been retained. In this concept, the populations can be viewed as distinct evolutionary units because they are evolving separately at the cytochrome *b* gene. At this time much more work into the degree of variability between these units is needed in order to determine their taxonomic rank.

Species Relationships

Within every tree constructed there has been a clear division of the two major clades in the genus *Vireo*: the eye-lined forms (*Vireosylva*) and the eye-ringed forms (*Vireo* and *Lanivireo*). This result perhaps is best illustrated by the number of transversions between and within the groups. The greatest number of transversions between species within a given clade is 6 while the number of transversions between the clades ranges from 5 to 11, averaging 8.2. These findings are consistent with the conventional treatment of this genus. Once again no strong support for the subgenus *Lanivireo* has been found. In all analyses its members are as closely related to species of the subgenus *Vireo* as they are to each other.

Within eye-lined Vireos, the branching order is identical in all cladograms (Figures 7,8,9 and 10) and the Fitch distance tree (Figure 6). The one exception is the placement of the Philadelphia Vireo in the Kitsch distance tree (Figure 5) which may be explained by a large accumulation of transitions (See next paragraph). The

phylogeny of the cladograms and the Fitch distance tree reveal a proto eye-lined Vireo having given rise to two forms; a proto red-eye and a *gilvus* form. The proto red-eye speciated further into *V. olivaceus* and *V. altiloquus*. Meanwhile, the proto *gilvus* form had given rise to the *V. gilvus ssp* and *V. philadephicus*. This evolution would be in support of Barlow's (1980) hypothesized species radiation. Here the postulated proto *Vireosylva* gave rise in Central America to the proto *gilvus* and proto red-eyed forms which subsequently invaded North America to establish breeding populations.

The relationship of *V. philadephicus* to *V gilvus ssp* is supported by all the cladograms and tenuously by the Fitch distance tree. However, as shown in Table 3, although there is on average 20 base pair differences between the Warbling and Philadelphia Vireos, there is only one transversional difference. This is in contrast to comparison between the two major lineages of the eye-lined Vireos presently put forward. Here 5 to 6 transversions have accumulated between them. It is not hard to imagine a scenario, especially at a transition to transversion bias of at least 10:1, in which transitions have randomly, but more rapidly than expected, accumulated in *V. philadephicus*. In this situation the Kitsch distance trees constructed with a 2:1 bias would, not unexpectedly, be different from the cladistic trees.

Previous work on the Philadelphia Vireo agrees with the phylogenetic relationship described here. Johnson *et al.* (1988) and Hamilton (1962) found *V. gilvus* and *V. philadelphicus* to be closely aligned in their analyses. The small Philadelphia (12 g) is within the size range of the Warbling Vireo and lacks the red iris of the larger (16 g)

Red-eyed Vireo. Behaviourially, the Philadelphia and Red-eyed Vireos are similar. They breed sympatrically in mixed boreal forests in which they display interspecific territoriality during the breeding season (Barlow and Rice 1977). Their mutually exclusive territories are maintained by similar song types which allow the smaller Philadelphia Vireo to compete successfully for space during the breeding season.

The close phylogenetic relationship of the phenotypically similar Red-eye and Black-whiskered Vireos is not surprising. DNA-DNA hybridization studies show these two species to be very closely related (Sibley and Ahlquist 1982). Together, these two species represent a breeding and migration continuum (Barlow 1980). Both species winter in northern South America. *Vireo altiloquus* breeds throughout the Caribbean including the coast of Florida while *V. olivaceus* breeds north and west of this range throughout North America and Central America.

The relationships of the taxa within the eye-ringed Vireos are less clear. The species radiation seen in Figure 10 appears more likely than that of Figure 9. In Figure 10 an ancestral proto eye-ringed Vireo has given rise to all subsequent forms. This evolution is favoured because of two points. First, it is the speciation predicted by the distance trees (Figures 5 and 6). Second this analysis weighted the data (8:1) closest to the estimated transversion to transition bias observed for the gene.

There appears to be two major groups within this clade: the *V. bellii, V. griseus* group and the *V. solitarius ssp*. The *V. solitarius ssp* clade is supported strongly in the

bootstrap analyses (76% Figure 5; 90% Figure 6; 81% Figure 7). The *V. bellii, V. griseus* clade is not as strongly supported by the bootstrap analyses (65% Figure 5; 44% Figure 6), but is found consistently in all trees but Figure 8. The relationships of the Black-capped (*V. atricapillus*) and Gray (*V. vicinior*) Vireos within this complex has the weakest bootstrap support. In most analyses, however, *V. vicinior* appears more closely aligned with the *bellii, griseus* group while *V. atricapillus* is closer to the *solitarius* group. The position of the root within this clade is unclear.

One of the clearer relationships within the eye-ringed clade is the association of the Bell's and White-eyed Vireos. This result supports relationships discussed by Hamilton (1962) and Orenstein and Barlow (1981) but is in direct conflict with those of Johnson *et al.* (1988). They reported *V. bellii* to be highly diverged from any other member of *Vireo*. These conflicting electrophoretic and DNA sequence results are both based on small sample sizes and justify further study into the protein and DNA variation of this taxon.

Generic Relationships

Except for two analyses, the Fitch distance tree and the unweighted cladogram, the genus *Vireo* appears to be monophyletic. In these two trees, *Cyclarhis gujanensis* is situated next to the *V. solitarius ssp.* Both these instances may be explained by convergence of transitional events. Neither branching pattern is not supported by bootstrap analysis (32% Figure 6; <50% Figure 7). It should be noted that no

members of the genus *Hylophilus* were examined in this study. This genus was also reported to be highly polyphyletic in the survey by Johnson *et al.* (1988) with its taxa grouping throughout the Family Vireonidae.

Monophyly is supported by all weighted cladograms and the Kitsch distance tree. This is in direct contrast to the results of Johnson *et al.* (1988) and in support of the traditional taxonomy. The weighted phylogenies are thought to provide the best estimates of the evolutionary relationships because they represent the data in the most realistic manner. These trees weight the mutational changes according to their probability of occurring. This weighting system is based on the premise that the slowly evolving changes contain a more consistent record of phylogeny because fewer parallel or reversal mutational events will have occurred. Convergence of character states will cause confusion in the phylogenetic analyses. Thus characters states that have a high probability of convergence (*i.e.* transitions) should be interpreted with caution.

Methodology

The low bootstrap confidences of the branches in the eye-ringed clade may be due to a number of methodological problems. First the length of sequence examined is only 273 bp. A study by Martin *et al.* (1990) investigated variation in genetic estimates in relation to the size of homologous sequence compared. They reported that variation in estimates of genetic distance increase as the area of homologous DNA examined

decreases. This fact may explain the difference in confidence levels between the eye-lined and eye-ringed clades. The eye-lined clade seems to contain two groups, the red-eyed and *gilvus* groups, which evolved relatively early in the history of the clade and then have only recently split into the present species examined here. The first split, characterized by at least 5 transversional events, followed by the more recent speciations of *V. gilvus* and *V. philadephicus*, characterized by 1 transversional event, and *V. olivaceus* and *V. altiloquus*, having no transversional events, are likely to be observable in analyses of small regions of DNA. Conversely, the eye-ringed clade seems to have undergone a more even speciation process. This is illustrated by a pairwise comparison of tranversional events between the member taxa which ranges evenly between 1 and 7. Since the evolution of this clade has been more uniform in nature, including a number of relatively contemporaneous speciation events, it is more likely that they will become blurred by the two processes of random parallel nucleotide substitution or reversions and the effect of examining a small region of homologous DNA. In order to test this hypothesis and improve the estimation of the phylogeny of the eye-ringed clade additional sequencing other regions of the mtDNA would be required.

Another technique-related problem that may have led to the low confidence values for the eye-ringed clade is the small sample sizes for each species. The seemingly contradictory characters that lead to low confidence values for the bootstrap analyses may be simply the retention of ancestral polymorphisms. Because the sample sizes presented are very low this possibility cannot be addressed. It seems unlikely,

however, as geographic variation in mtDNA has been shown to be low in other North American birds (Ball *et al.* 1988; Moore *et al.* 1991).

A final technical problem with the study is that only 9 of the 26 recognized *Vireo* species were included in this survey. The addition of the remaining *Vireo* species would help to clarify some species relationships. One of the strengths of comparing sequence data is that samples once obtained and sequenced can be added easily to the data base for reanalysis. This is clearly a future direction for investigation of vireo systematics.

Utility of Cytochrome b for Phylogenetic Inference

The amount of variation present in the portion of cyt-*b* sequence analyzed has proven sufficient for an estimation of *Vireo* phylogeny. This type of survey is suitable for population level studies ranging from generic (Irwin *et al.* 1991) to subspecific (Edwards and Wilson 1990; this study) comparisons. The low amount of variation observed within subspecies, which may be indicative of the entire mitochondrial genome in birds, would not recommend a cyt-*b* comparison for lower level comparisons.

For use in subspecific comparison, cyt-*b* is useful in the identification of different mitochondrial haplotypes if they exist and in estimating genetic distances between them. However, it is not very informative about population interactions between the

subspecies. Possible haplotype introgression can be revealed suggesting hybridization but other observational or nuclear DNA data are needed to substantiate the claim. This is a drawback not only of cyt-*b* data but obviously of all mtDNA analyses since only a single maternal marker is present in the offspring.

The rate of nucleotide substitution in cyt-*b* shows a bias of at least 10:1 to transitional events. This fact should be noted when interpreting results. The larger the relative evolutionary time between comparison of species the higher the likelihood of parallel or reversal nucleotide substitutions which confuse phylogenetic relationships. This problem is obviously great when analyzing the rapidly accumulating transitions. Thus, the slower accumulating transversions should contain a more accurate record of phylogeny and should be weighted accordingly.

Cytochrome b Evolution

In total 12 amino acid changes were observed to have occurred in the region of cyt-b examined. All of these changes appear to be conservative and probably have no functional significance. These 12 amino acid changes are caused by 16 nucleotide substitutions. In comparison to the 80 silent nucleotide substitutions the low number of amino acid changes would seem to indicate that the gene is under a high selective pressure. A high selective presure would agree with the importance of the molecule in the proper transfer of electrons in the electron transport chain; a process essential for energy production.

Conclusion

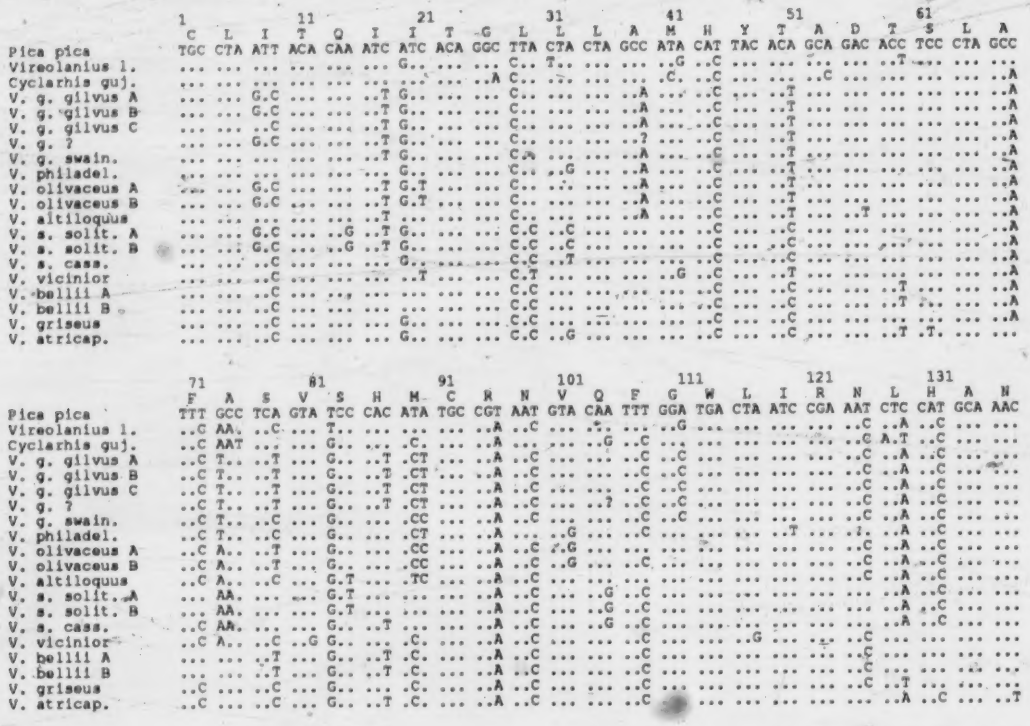
In summary cytochrome *b* sequence analysis has proven a useful tool for a reconstruction of the phylogenetic history of the genus *Vireo*. The results indicate that the genus is a monophyletic taxon. *Vireo* is made up of two major lineages: the eye-lined vireos (subgenus *Vireosylva*) and the eye-ringed vireos (subgenera *Vireo* and *Lanivireo*). At this time I would, in contrast to Johnson *et al.*, 1988., still recommend the use of part of the subgeneric classification. The subgenus *Lanivireo* should not be used and its members placed within the Subgenus *Vireo*. The relationships between the members of the eye-lined clade are clear, however, the relationship between members of the eye-ringed clade is less so. This situation may be rectified by the sequencing of additional regions of DNA, the increase of present sample sizes or the addition of more *Vireo* species to the present survey. The analysis of subspecific variation has revealed the presence of distinct mtDNA haplotypes forms and clearly justifies the continued investigation into the rank of the these taxa.

Table 1: Sample location, size and cyt-b haplotypes of the taxa studied.

Taxa	Sample collection location	N	Haplotypes
Family Vireonidae			
Subfamily Vireoninae			
Genus *Vireo*			
Eye-ringed vireos			
Subgenus *Vireo*			
Bell's Vireo			
Vireo bellii bellii	Tom Green Co., Texas	1	Vbe A
	Cleveland Co., Oklahoma	1	Vbe B
White-eyed Vireo			
Vireo griseus	Brewster Co., Texas	1	Vgr
Black-capped Vireo			
Vireo atricapillus	Kerr Co., Texas	1	Vat
Subgenus *Lanivireo*			
Solitary Vireo			
Vireo solitarius	Jackfish Lake, Sask.	1	Vss A
solitarius	Seven Mile Creek, Alta.	1	Vss B
	Nordegg, Alta.	1	Vss B
	Clearwater River, Alta.	1	* Vsc
Vireo s. cassinii	Windsor Ridge, Alta.	3	Vsc (3)
	Hailstone Butte, Alta.	1	Vsc
	Seven Mile Creek, Alta.	1	Vsc
Grey Vireo			
Vireo vicinior	Brewster Co., Texas	1	Vvi
Eye-lined vireos			
Subgenus *Vireosylva*			
Philadelphia Vireo			
Vireo philadelphicus	Weyakwin, Sask.	1	Vph
Red-eyed Vireo			
Vireo olivaceus	Weyakwin, Sask.	1	Vol A
	Winefred Lake, Alta.	2	Vol B (2)
Black-whiskered Vireo			
Vireo a. altiloquus	Jamaica	1	Val
Warbling Vireo			
Vireo gilvus gilvus	North Battleford, Sask.	2	Vgg B, Vgg C
	Beaverhills Lake, Alta.	3	Vgg A (2), * Vgs
Vireo g. swainsonii	Castle River, Alta.	2	Vgs (2)
	Hailstone Butte, Alta.	2	Vgs (2)
	Cypress Hills, Alta.	1	Vgs
	Sundre, Alta.	1	* Vg ?
Subfamily Cyclarhinae			
Genus *Cyclarhis*			
Rufous-browed Peppershrike			
Cyclarhis gujanensis	Venezuela	1	C.gu
Subfamily Vireolaninae			
Genus *Vireolanius*			
Slaty-capped Shrike-vireo			
Vireolanius leucotis	Venezuela	1	Vl.le
Family Corvidae			
Black-billied Magpie			
Pica pica	Edmonton, Alta.	1	P.pi
	Totals	N=32	Haplotypes = 20

An '*' indicates the occurrence of a haplotype most commonly associated with the other subspecies. Bracketed numbers indicate number of samples with the given haplotype at each location. Haplotype sequence data is found in Table 2 (for example, Vgg b = V.g.gilvus B).

Table 2: Cytochrome *b* haplotype sequence data.

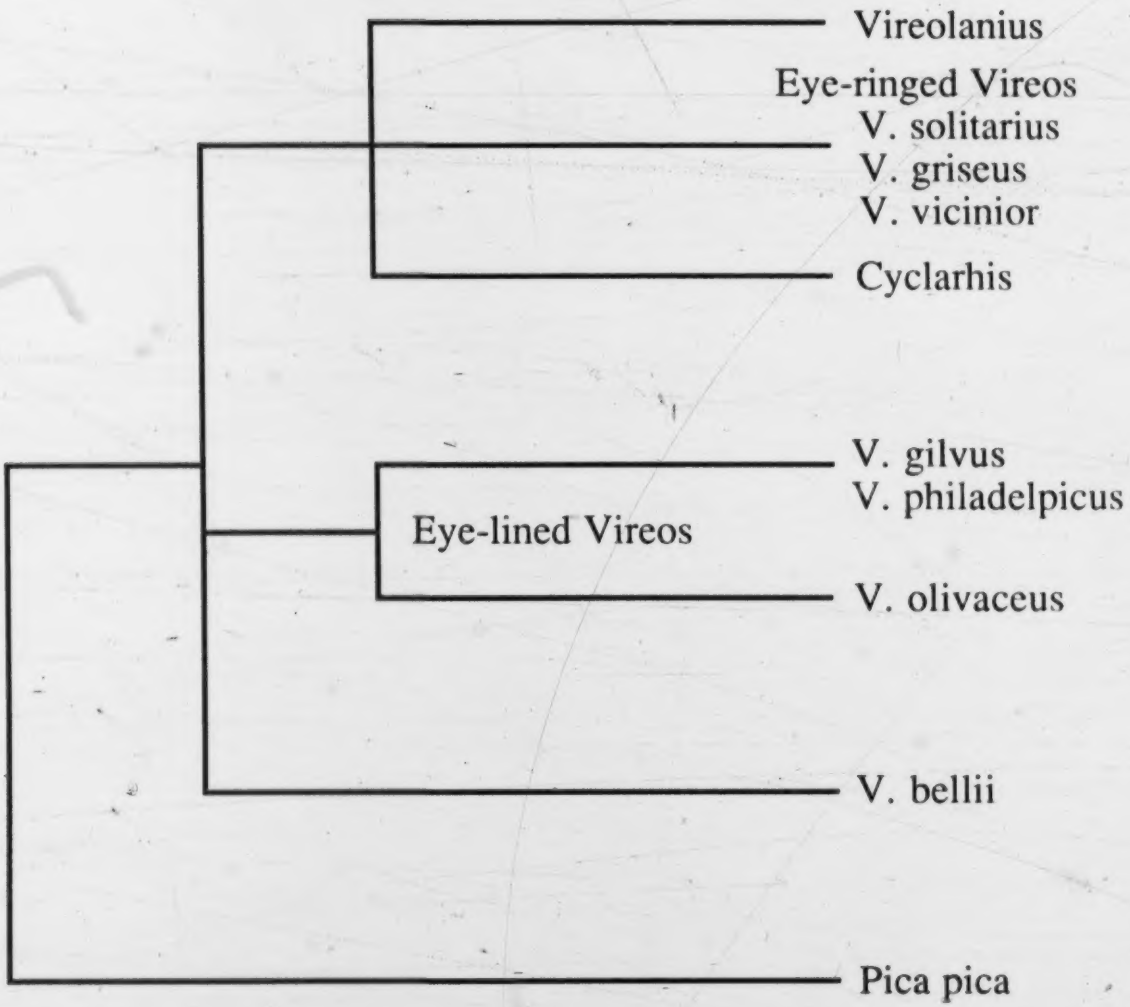


Identical base pairs are indentified by '.' , a change is represented by a letter (A=adenine, C=cytosine, G=guanine and T=thymine) and ambiguities by '?'.

Table 3: Pairwise estimates of Kimura's distance (sequence divergence between haplotypes) based on Kimura's `2-parameter ' model (1980) above the diagonal. Total number of base pair substitutions followed by number of transversions below the diagonal.

Haplo	Vgg A	Vgg B	Vgg C	Vgs	Vol A	Vol B	Vss A	Vss B	Vsc	Vvi	Vph	Vbe A	Vbe B	Vgr	Val	Vat	Vl.le	C.gu	P.pi
Vgg A	-	.0037	.0037	.0298	.0575	.0496	.1075	.1159	.0991	.1072	.0805	.0947	.0947	.0944	.0815	.1075	.1345	.1171	.1545
Vgg B	1|0	-	.0074	.0336	.0614	.0535	.1117	.1202	.1033	.1114	.0845	.0988	.0988	.0986	.0855	.1117	.1389	.1215	.1591
Vgg C	1|0	2|0	-	.0260	.0614	.0535	.1117	.1202	.0949	.1030	.0765	.0905	.0905	.0903	.0774	.1033	.1301	.1215	.1500
Vgs	8|0	9|0	7|0	-	.0614	.0535	.1075	.1159	.0991	.0947	.0725	.1072	.1072	.0903	.0614	.1117	.1301	.1258	.1455
Vol A	15|5	16|5	16|5	16|5	-	.0074	.1069	.1153	.1069	.0978	.0976	.0978	.0978	.0894	.0450	.1148	.1288	.1165	.1447
Vol B	13|5	14|5	14|5	14|5	2|0	-	.1069	.1153	.0986	.0896	.0976	.0896	.0896	.0813	.0450	.1064	.1288	.1080	.1492
Vss A	27|11	28|11	28|11	27|11	28|9	28|9	-	.0074	.0489	.1017	.1284	.1022	.1022	.1061	.1111	.1187	.1414	.1156	.1623
Vss B	29|11	30|11	30|11	29|11	30|9	30|9	2|0	-	.0489	.1017	.1284	.1106	.1106	.1061	.1196	.1187	.1371	.1199	.1714
Vsc	25|11	26|11	24|11	25|11	27|9	25|9	13|0	13|0	-	.0935	.1199	.0776	.0696	.0734	.1027	.0855	.1072	.0947	.1488
Vvi	26|10	27|10	25|10	24|10	24|6	22|6	26|5	26|5	23|5	-	.1111	.0807	.0807	.0765	.1061	.0971	.1281	.1202	.1476
Vph	21|1	22|1	20|1	19|1	25|5	25|5	32|10	31|10	31|10	29|9	-	.1281	.1281	.1193	.1142	.1030	.1562	.1473	.1541
Vbe A	24|10	25|10	23|10	27|10	24|6	24|6	26|7	28|7	20|6	21|2	32|9	-	.0074	.0568	.0978	.0889	.1288	.1117	.1307
Vbe B	24|10	25|10	25|10	27|10	24|6	24|6	26|7	28|7	18|6	21|2	33|9	2|0	-	.0568	.0978	.0809	.1288	.1117	.1307
Vgr	24|9	25|9	23|9	23|9	23|5	21|5	27|5	27|5	19|6	20|2	30|8	15|1	15|1	-	.0976	.0932	.1199	.0905	.1304
Val	21|6	22|6	20|6	16|6	11|0	11|0	29|9	31|9	27|9	27|6	29|5	25|6	25|6	25|5	-	.1232	.1033	.1295	.1270
Vat	27|11	28|11	26|11	29|11	27|7	25|7	30|5	30|5	21|6	25|3	26|10	22|3	20|3	34|4	30|7	-	.1321	.1562	.1524
Vl.le	33|14	34|14	32|14	32|16	31|12	29|12	35|10	34|10	30|10	33|9	38|14	31|9	31|9	30|10	28|11	32|8	-	.1335	.1253
C.gu	29|15	30|15	30|15	31|15	29|13	27|13	31|11	32|11	24|10	30|11	36|14	27|11	27|11	23|10	32|13	37|14	33|12	-	.1488
P.pi	36|16	37|16	37|16	35|14	36|14	36|14	40|13	42|13	36|12	36|10	38|15	31|12	31|12	32|10	31|14	36|11	32|9	37|13	-

Figure 1: Diagrammatic representation of the main results of Johnson *et al.*, 1988 on the species relationship within the Family Vireonidae. The species relationships of Johnson *et al.* are based on UPGMA phenograms and Wagner trees generated from Rogers' genetic distances. Genetic distances were estimated from the protein electrophoretic variation of 29 genetic loci. In this Figure no species of the genus *Hylophilus* were included because they were not analyzed in present analysis. Johnson *et al.*, 1988, reported this taxon to be highly polyphyletic with species branching throughout the tree shown.



Diagramatic Representation of the results of Johnson et al. 1988

Figure 2: Diagrammatic representation of avian mitochondrial DNA and the cytochrome *b* gene. The partial map of mtDNA is based on the Chicken mtDNA (Desjardins and Morais 1990). The gene for cytochrome *b* contains no introns and codes for 380 amino acids. The hatched area represents the region sequenced for this study.

Cytochrome b

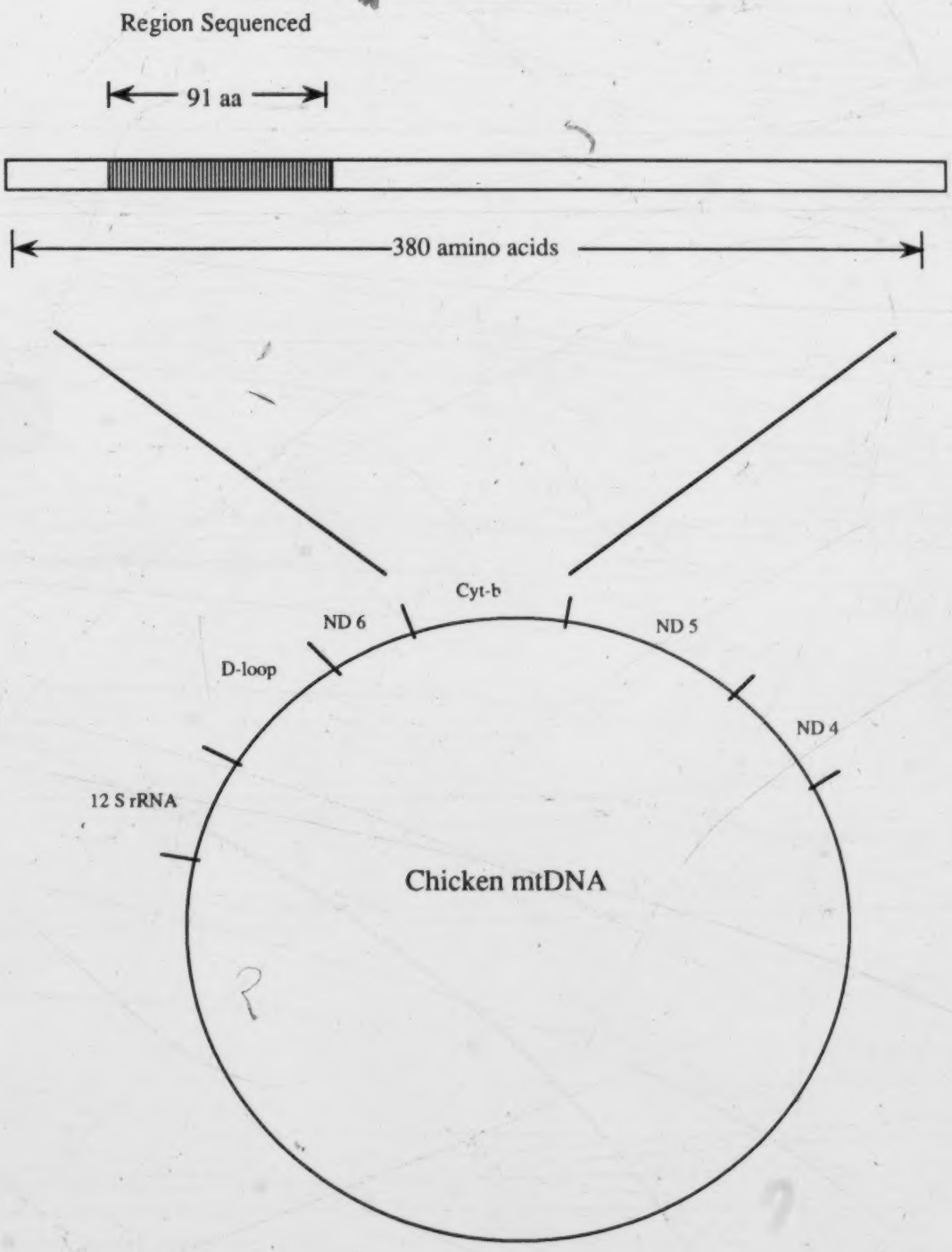


Figure 3: Location of the collection sites of the Warbling Vireo (*Vireo gilvus ssp*) in Alberta and Saskatchewan. *V. g. swainsonii* breeding populations are found in the lined area and in the Cypress Hills. *V. g. gilvus* breeding populations are found in the doted area. A '?' indicates Warbling Vireo have not been found in these areas despite repeated collection attempts. Maps are based on the Provincial Museum of Alberta's collection records (W.B. McGillivray, unpublished data). Birds morphologically typed *V. g. swainsonii* were collected in Sundre (Sun), Hailstone Butte (HaB), Cypress Hills (CyH) and along the Castle River (CaR). Birds morphologically typed *V. g. gilvus* were collected in Beaverhills Lake (BhL) and North Battleford (NrB). A closed circle indicates that only the haplotype most commonly associated with *V. g. swainsonii* populations was found at this location. A dotted circle indicates that only haplotypes most commonly associated with *V. g. gilvus* populations were found. A meshed circle indicates that both haplotypes were present in separate samples at the given location.

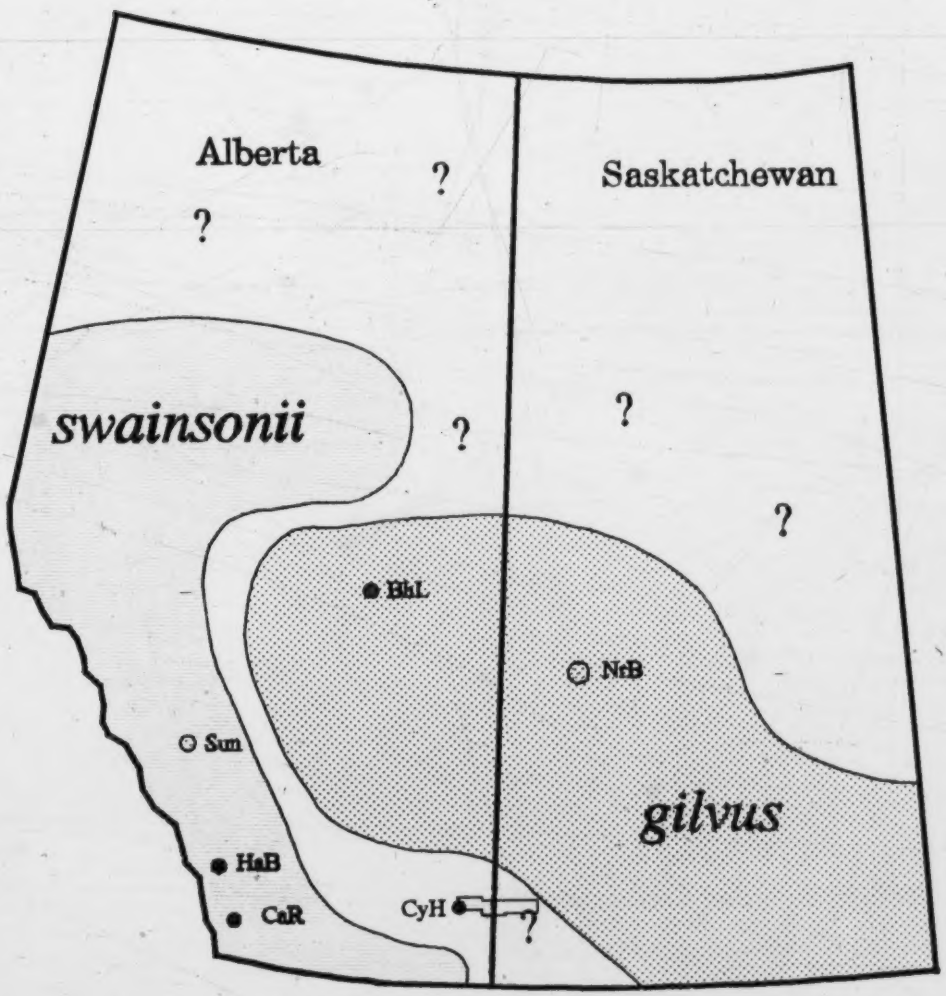

Alberta
?
?
Saskatchewan
swainsonii
?
?
?
BkL
NrB
Sun
gilvus
HaB
CaR
CyH
?

Figure 4: Location of the sample collection sites of the Solitary Vireo (*Vireo solitarius ssp*) in Alberta and Saskatchewan. Breeding *V. s. cassinii* populations in the lined area line while breeding *V. s. solitarius* populations are found in the doted area. Maps are based on the Provincial Museum of Alberta's collection records (W.B. McGillivray, unpublished data). Samples morphologically typed *V. s. cassinii* were collected in Hailstone Butte (HaB), along the Castle River (CaR) and Seven mile Creek (SmC). Samples morphologically typed *V. s. solitarius* were collected by Jackfish Lake (JcL), Nordegg (Nor), the Seven mile Creek (SmC) and the Clearwater River (ClR). A closed circle indicates the presence of only the *V. s. cassinii* haplotype at any location. A dotted circle indicates the presence of only *V. s. solitarius* haplotypes forms at the given location. A meshed circle indicates that both haplotypes forms are found in separate samples at the given location.

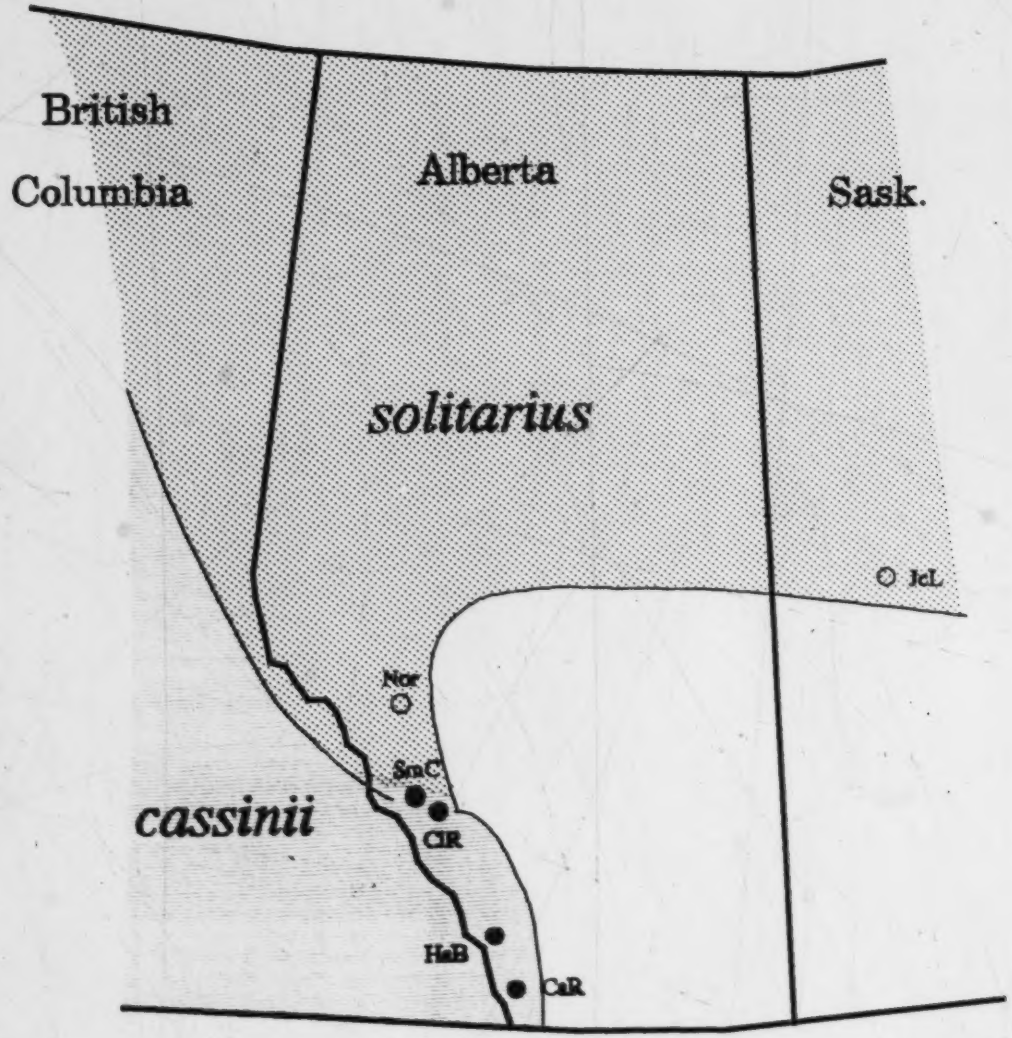

British
Columbia
Alberta
Sask.
solitarius
JcL
Nor
SmC
cassinii
ClR
HaB
CaR

Figure 5: Fitch-Margoliash distance tree constructed under the assumption that an evolutionary clock is in effect. The tree was constructed from the pairwise distance estimates in Table 2 utilizing the computer program Kitsch contained in the phylogenetic package Phylip 3.3 (Felsenstein 1990). The branch length to the node indicates the percent divergence (x100) of the haplotypes. Numbers located at the nodes indicate the number bootstrap trees out of 100 that contain this branching pattern. An '*' indicates this node was not found in the majority rule tree of the bootstrap analysis.

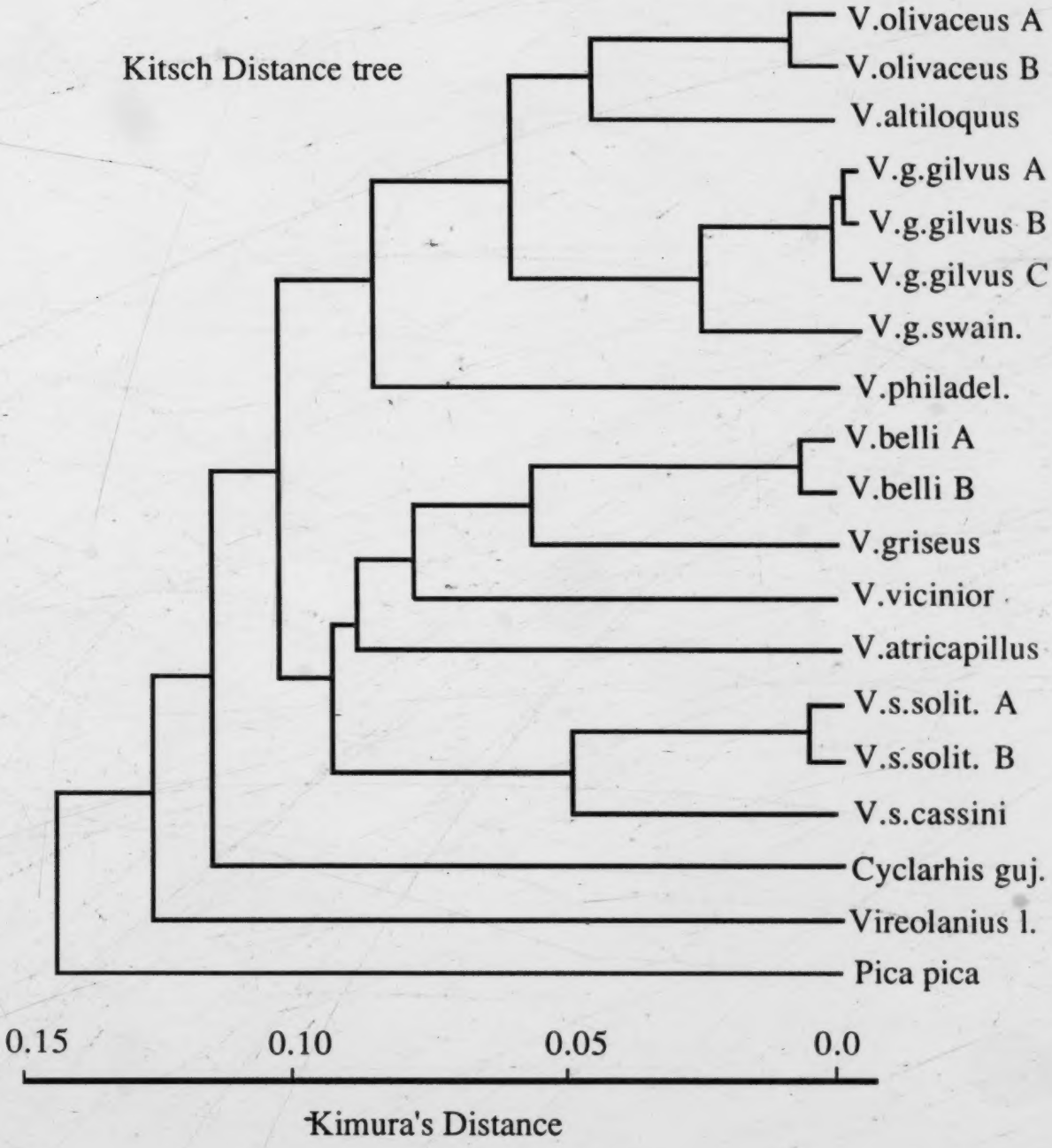

Kitsch Distance tree
V.olivaceus A
V.olivaceus B
V.altiloquus
V.g.gilvus A
V.g.gilvus B
V.g.gilvus C
V.g.swain.
V.philadel.
V.belli A
V.belli B
V.griseus
V.vicinior
V.atricapillus
V.s.solit. A
V.s.solit. B
V.s.cassini
Cyclarhis guj.
Vireolanius l.
Pica pica
0.15
0.10
0.05
0.0
Kimura's Distance

Figure 6: Fitch-Margoliash distance tree. The tree was constructed from the pairwise distance estimates in Table 2 utilizing the computer program Fitch contained in the phylogenetic package Phylip 3.3 (Felsenstein 1990). Per cent divergence is represented by the total horizontal distance between terminal tips and can be estimated using the scale. The number next to each node is the number of bootstrap out of 100 that contain this branching pattern. An '*' indicates the node was not found in the majority rule bootstrap consensus tree.

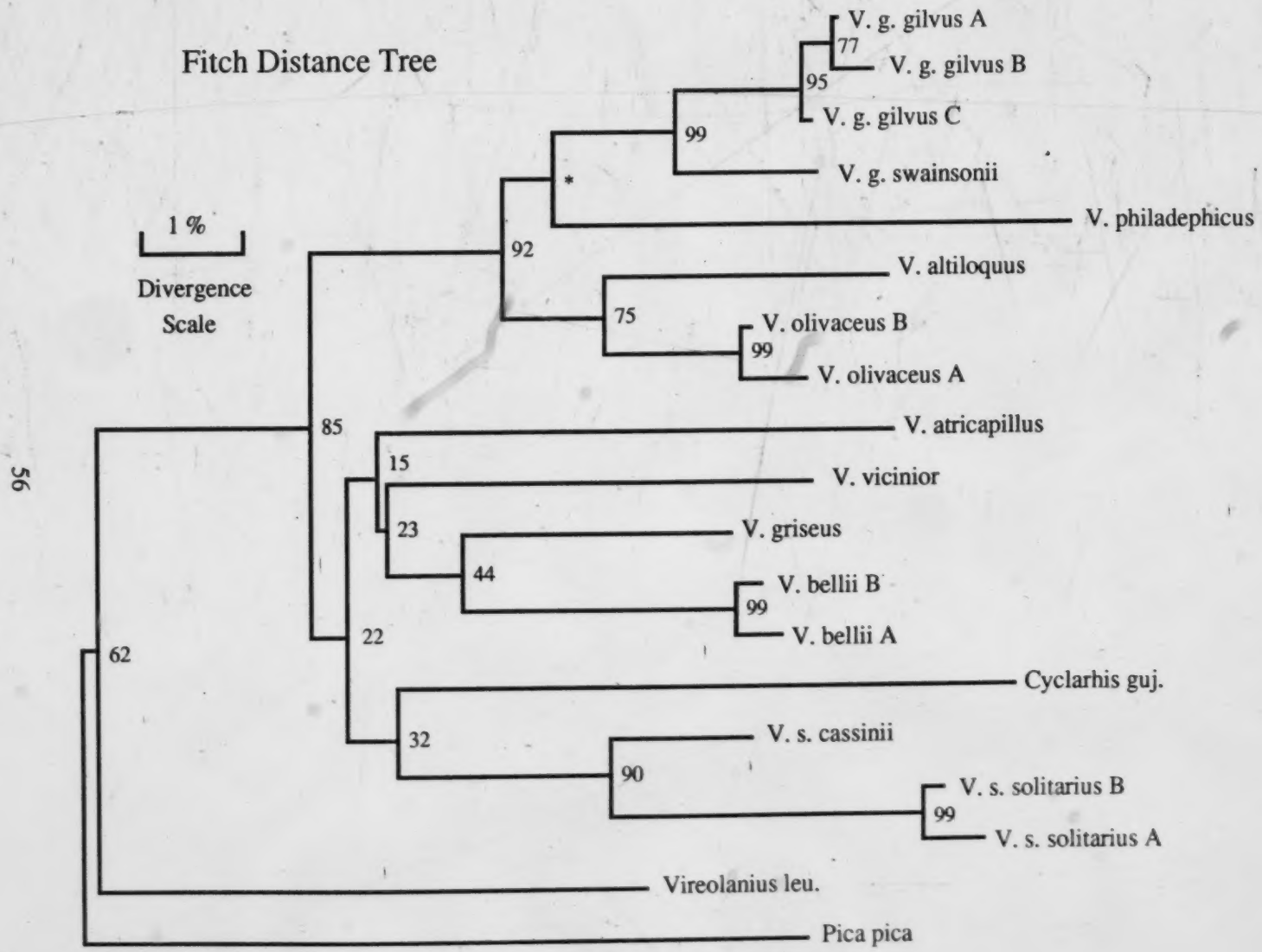

Fitch Distance Tree
1 %
Divergence
Scale
V. g. gilvus A
77
V. g. gilvus B
95
V. g. gilvus C
99
V. g. swainsonii
*
V. philadephicus
92
V. altiloquus
75
V. olivaceus B
99
V. olivaceus A
85
V. atricapillus
15
V. vicinior
23
V. griseus
44
V. bellii B
99
V. bellii A
22
62
Cyclarhis guj.
32
V. s. cassinii
90
V. s. solitarius B
99
V. s. solitarius A
Vireolanius leu.
Pica pica

Figure 7: The majority rule consensus cladogram constructed from all variable sites. Constructed utilizing the heuristic search method of the computer program Paup 3.0 (g) (Swofford 1989). For this analysis all characters received equal weight. This tree was constructed from 43 trees requiring 186 steps or less (4 trees conforming to this consensus tree were found at 185). The number along the branch indicates the percent of trees containing this branching pattern. The numbers at the nodes indicate the level of significance (out of 100) of the branching patterns as found in a bootstrap analysis constructing 100 trees. Branch nodes without a number were not supported in at least 50% of the bootstrap trees.

Unweighted Cladogram

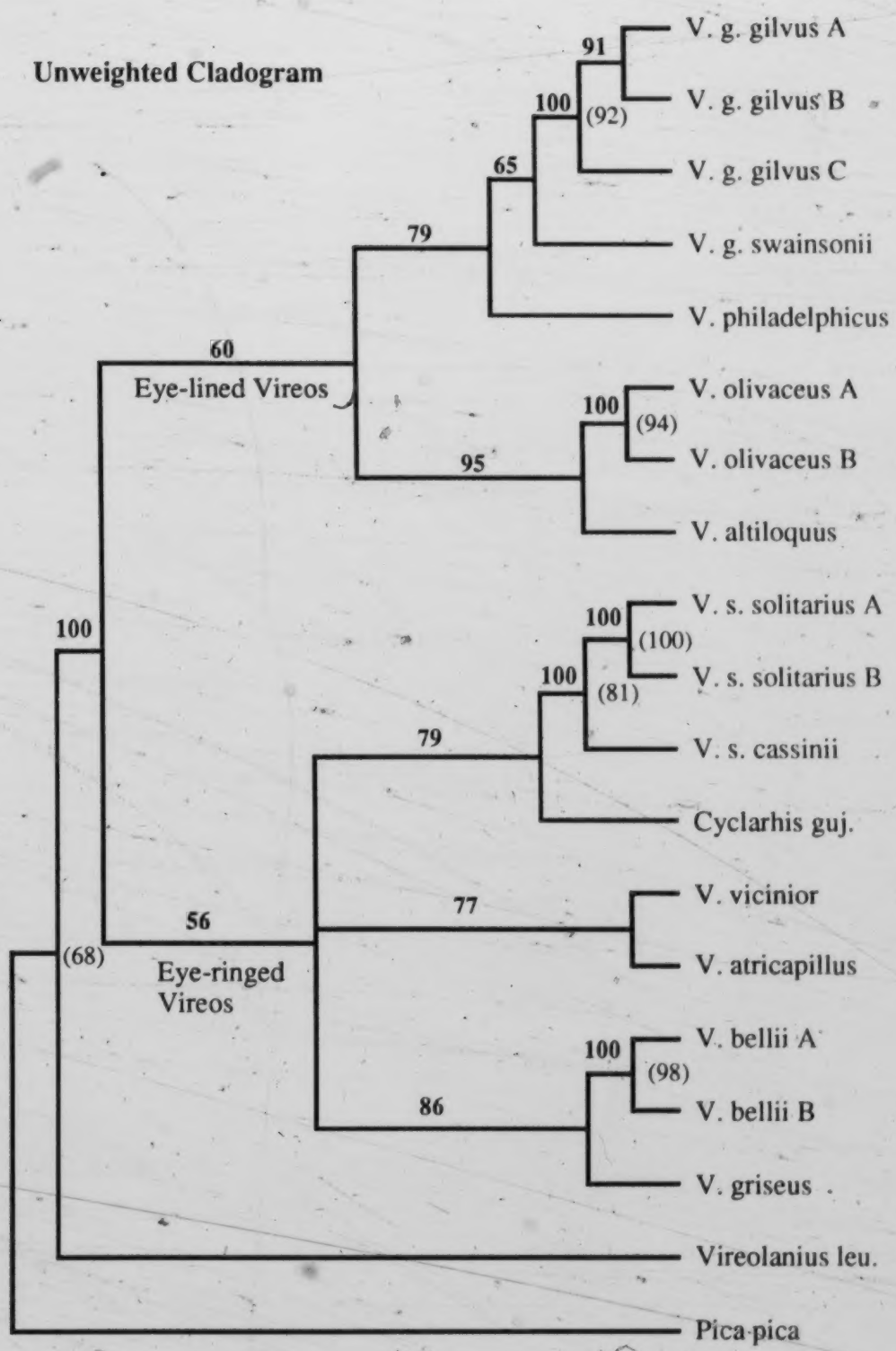


Figure 8: The majority rule consensus cladogram derived from phylogenetically informative transversions and selected 1st and 2nd codon base pair changes. The branch-bound search method of PAUP 3.0 (g) (Swofford 1989) was used to find 26 equally parsimonious trees requiring 37 mutational steps or less (1 tree was found to require 36 steps). The bold numbers along the branches indicate the percentage of tree that possess the given branch point. The bracketed numbers at the nodes indicate the percentage of bootstrap trees (out of 400) that contain the given branch point. Nodes without numbers indicate that the branch point was not found in at least 50% of the bootstrap trees.

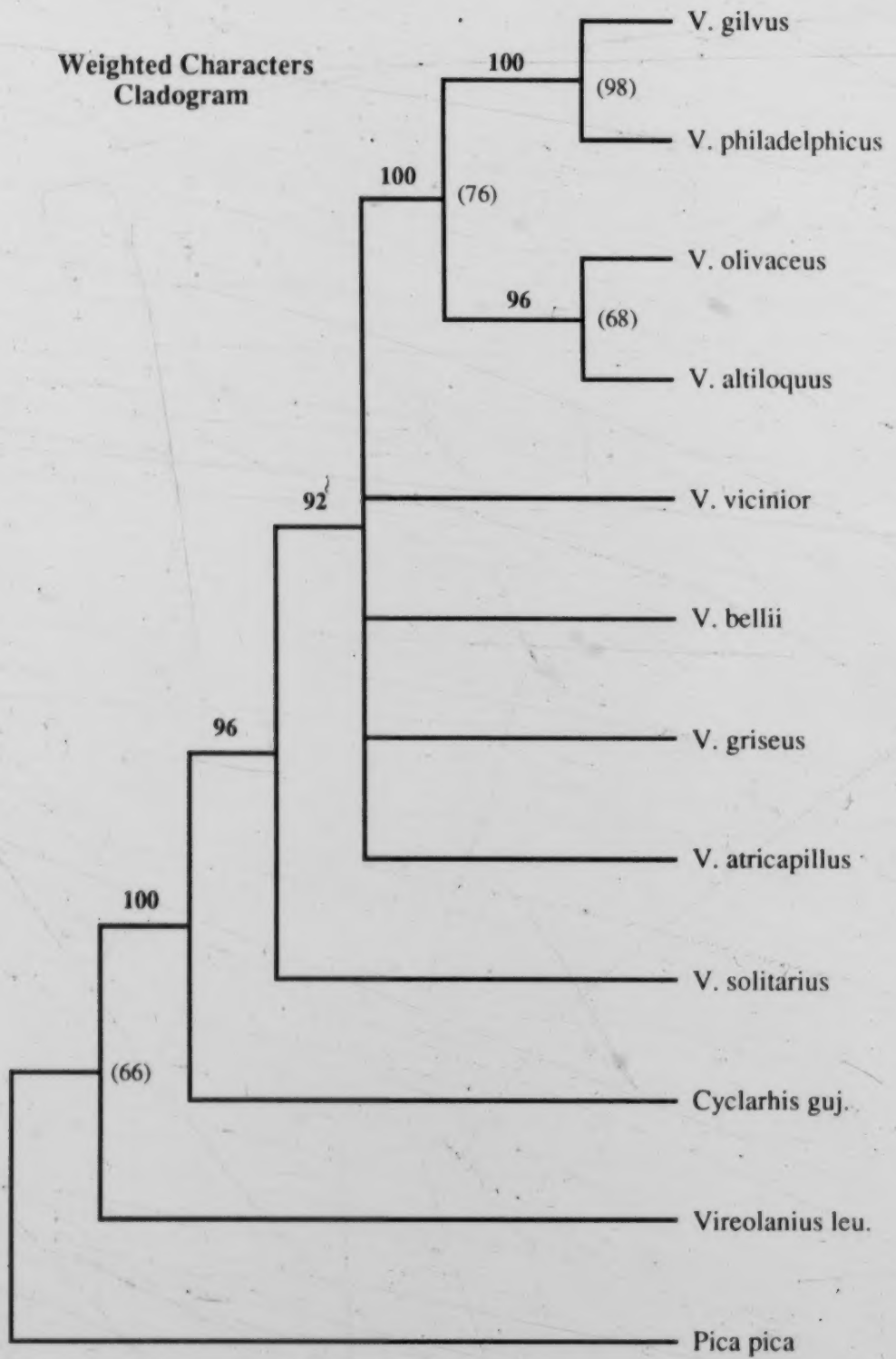

Weighted Characters
Cladogram
V. gilvus
100
(98)
V. philadelphicus
100
(76)
V. olivaceus
96
(68)
V. altiloquus
92
V. vicinior
V. bellii
96
V. griseus
V. atricapillus
100
V. solitarius
(66)
Cyclarhis guj.
Vireolanius leu.
Pica pica

Figure 9: The single most parsimonious tree (i.e. shortest total length) found when transversional and the selected 1st and 2nd codon base pairs changes within the matrix used to construct Figure 7 were given a weight of 2. This tree was revealed by utilizing the branch-bound search procedure of the phylogenetic computer program PAUP 3.0 (g) (Swofford 1989). It requires 186 mutational steps. Species are indentified to subgenera by the following letters in brackets; (VS) *Vireosylva*, (V) *Vireo* and (LV) *Lanivireo*.

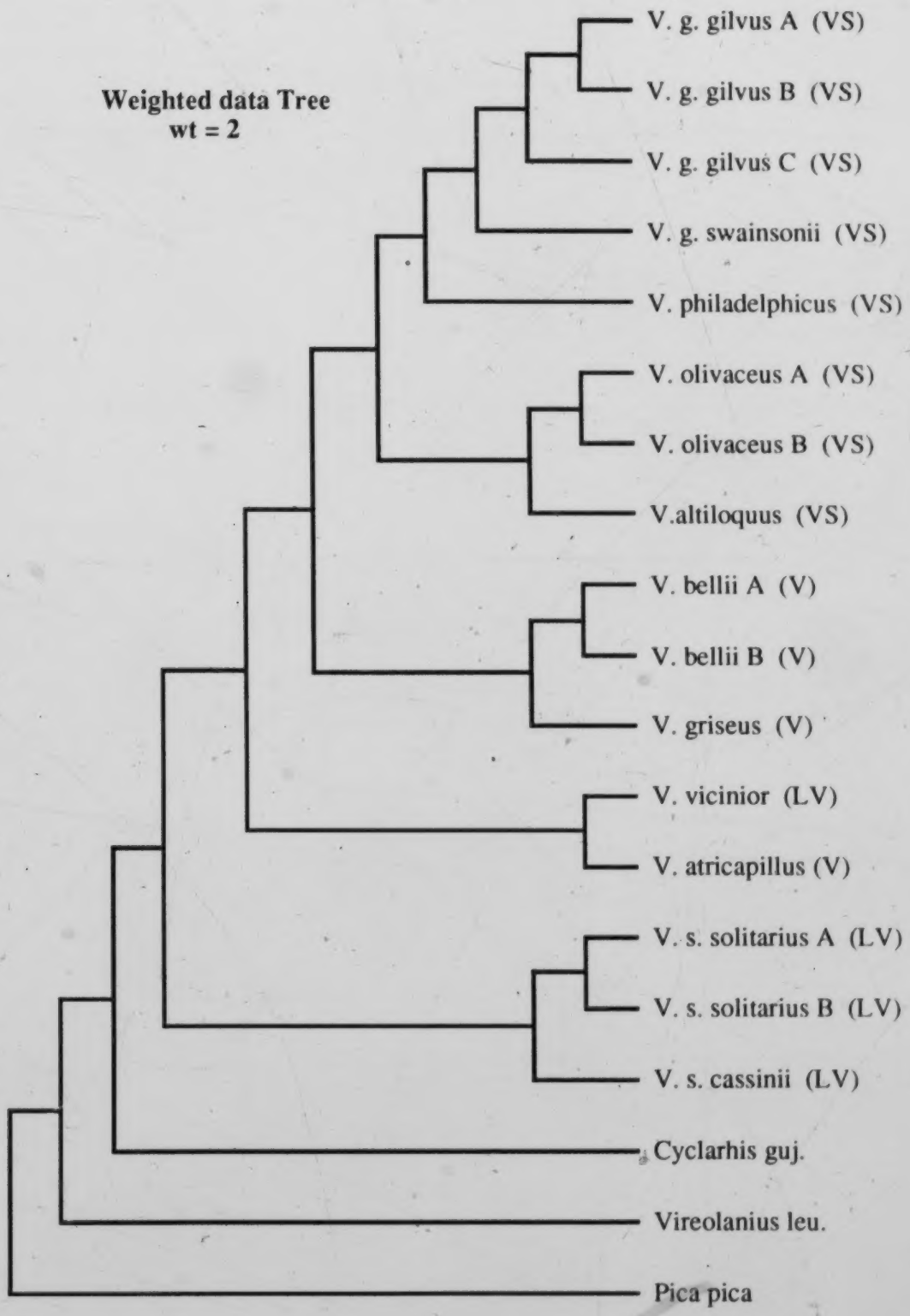

Weighted data Tree
wt = 2
V. g. gilvus A (VS)
V. g. gilvus B (VS)
V. g. gilvus C (VS)
V. g. swainsonii (VS)
V. philadelphicus (VS)
V. olivaceus A (VS)
V. olivaceus B (VS)
V.altiloquus (VS)
V. bellii A (V)
V. bellii B (V)
V. griseus (V)
V. vicinior (LV)
V. atricapillus (V)
V. s. solitarius A (LV)
V. s. solitarius B (LV)
V. s. cassinii (LV)
Cyclarhis guj.
Vireolanius leu.
Pica pica

Figure 10: The single most parsimonious tree (i.e. shortest total length) when transversional and selected 1st and 2nd codon base pair changes within the matrix used to construct Figure 7 were given weights of 4 and 8 respectively. This tree was found utilizing the branch-bound search procedure of the phylogenetic computer program PAUP 3.0 (g) (Swofford 1989). It requires 187 mutational steps. Species are indentified to subgenera by the following letters in brackets; (VS) *Vireosylva*, (V) *Vireo* and (LV) *Lanivireo*.

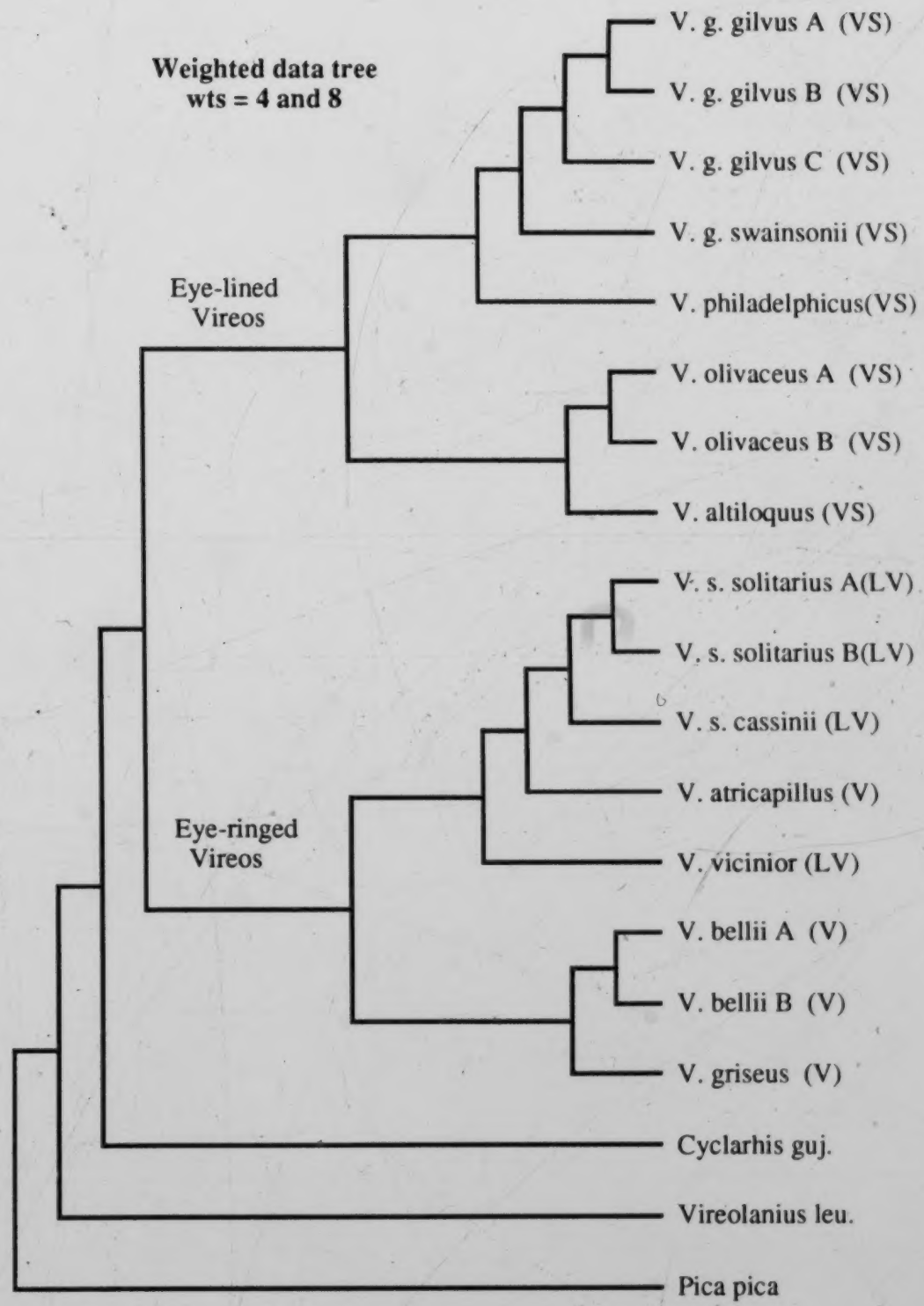
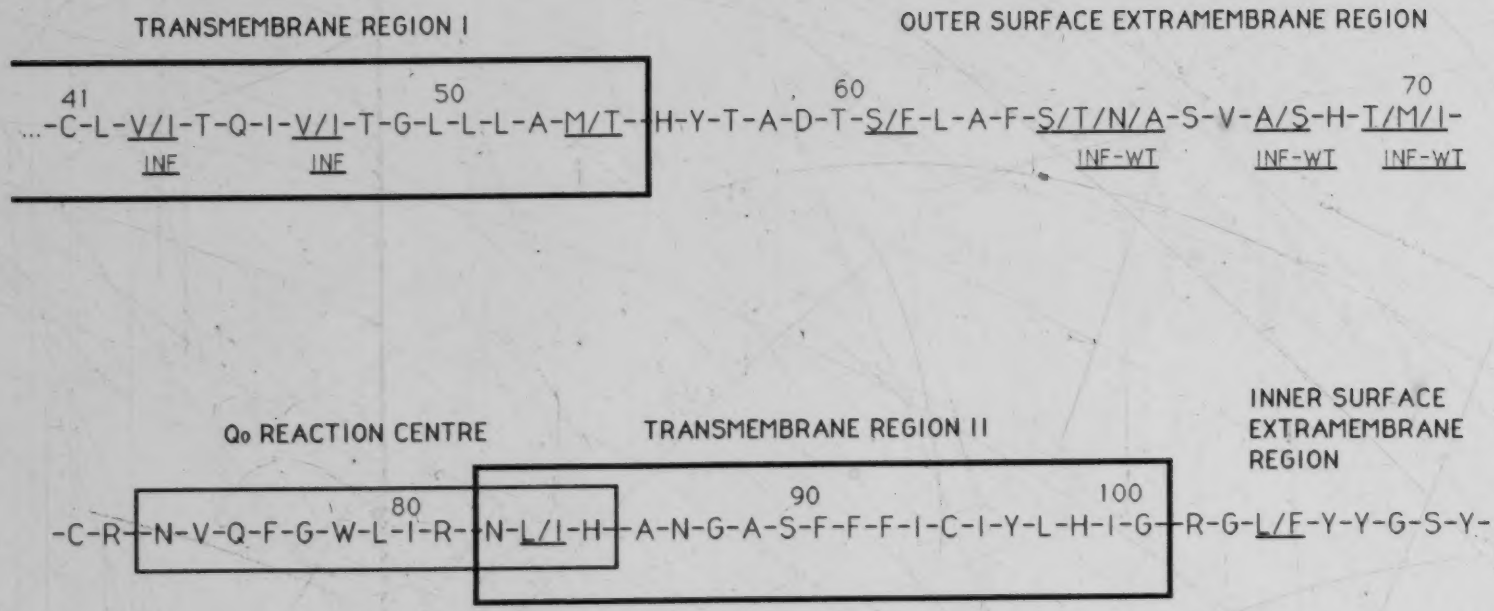

Weighted data tree
wts = 4 and 8
V. g. gilvus A (VS)
V. g. gilvus B (VS)
V. g. gilvus C (VS)
V. g. swainsonii (VS)
V. philadelphicus(VS)
Eye-lined
Vireos
V. olivaceus A (VS)
V. olivaceus B (VS)
V. altiloquus (VS)
V. s. solitarius A(LV)
V. s. solitarius B(LV)
V. s. cassinii (LV)
V. atricapillus (V)
Eye-ringed
Vireos
V. vicinior (LV)
V. bellii A (V)
V. bellii B (V)
V. griseus (V)
Cyclarhis guj.
Vireolanius leu.
Pica pica

Figure 11: The amino acid sequence variation of the region of cytochrome *b* analyzed in relationship to putative transmembrane and Q_0 reaction regions (Howell 1989). Twelve variable amino acid sites are identified by underlining. At most 4 and usually 2 amino acid were variable at these sites. Sites with INF underneath are phylogenetically informative. Sites with INF-WT underneath are the weighted informative sites used to construct Figures 9 and 10. The numbers correspond to homologous sequence of chicken mitochondrial cytochrome *b* (Desjardins and Morais 1990).


TRANSMEMBRANE REGION I
OUTER SURFACE EXTRAMEMBRANE REGION
41
50
60
70
...-C-L-V/I-T-Q-I-V/I-T-G-L-L-L-A-M/T-H-Y-T-A-D-T-S/F-L-A-F-S/T/N/A-S-V-A/S-H-I/M/I-
INF
INF
INF-WI
INF-WI
INF-WI
Qo REACTION CENTRE
TRANSMEMBRANE REGION II
INNER SURFACE EXTRAMEMBRANE REGION
80
90
100
-C-R-N-V-Q-F-G-W-L-I-R-N-L/I-H-A-N-G-A-S-F-F-F-I-C-I-Y-L-H-I-G-R-G-L/F-Y-Y-G-S-Y-
TRANSMEMBRANE REGION III
110
120
130
-L-N-K-E-T-W-N-V/I-G-V-I-L-L-L/F-T/A-L-M-A-T-A-F-V-G-...
INF

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Appendix A: Solution Information

Total DNA Extraction

L-Medium	0.10 M	EDTA
	0.10 M	Tris
	0.20 M	NaCl

* Phenol/Chloroform/Isoamyl Alcohol

25 : 24 : 1
Equilibrate with 0.1M Tris.HCl (ph 7.6)

mtDNA Extraction

H-Medium	10 mM	EDTA
	30 mM	Tris
	0.25 M	Sucrose
Solution I	10 mM	EDTA
	10 mM	Tris
	0.15 M	NaCl
Solution II	0.2 N	NaOH
	1%	SDS
Solution III	60 ml	5M Potassium Acetate
	11.5 ml	Glacial Acetic Acid
	28.5ml	H_2O

Resulting solution is 3M with respect to Potassium and 5M to acetate

PCR amplification

Standard PCR Buffer (10x)

20 mM	$MgCl_2$
0.50 M	KCl
0.10 M	Tris
1 mg/ml	BSA

Appendix A: cont.

Asymmetric PCR Buffer (10x)

5 mM	$MgCl_2$
0.50 M	KCl
0.10 M	Tris
1 mg/ml	BSA

Other buffers

* TBE (0.5 X)

0.045 M	Tris.borate
0.001 M	EDTA

* TAE (1 X)

0.04 M	Tris.acetate
0.001 M	EDTA

* TE (1 X)

10 mM	Tris.hcl (ph 8.0)
1 mM	EDTA

* Agarose Gel Loading Buffer (6X)

0.25%	bromophenol blue
0.25%	xylene cyanol
30%	glycerol in H_2O

DNA Sequencing — All solutions for sequencing were supplied in the following kits. See manuals for solution information.

United States Biochemical

Step-by-Step Protocols for DNA Sequencing with Sequenase Version 2.0 (4th ed., 1990)

or

Pharmacia LKB Biotechnology

T7Sequencing Kit Instructions (1990)

* indicates solution information taken from Sanbrook et al. (1990)